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Suicide Inactivation of Bacterial Cystathionine γ-Synthase and Methionine γ-Lyase during Processing of L-Propargylglycine†

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ABSTRACT: L-Propargylglycine, a naturally occurring γ,δ-acetylenic α-amino acid, induces mechanism-based inactivation of two pyridoxal phosphate dependent enzymes of methionine metabolism: (1) cystathionine γ-synthase, which catalyzes a γ-replacement reaction in methionine biosynthesis, and (2) methionine γ-lyase, which catalyzes a γ-elimination reaction in methionine breakdown. Biphasic pseudo-first-order inactivation kinetics were observed for both enzymes. Complete inactivation is achieved with a minimum molar ratio ([propargylglycine]/[enzyme monomer]) of 4:1 for cystathionine γ-synthase and of 8:1 for methionine γ-lyase, consistent with a small number of turnovers per inactivation event. Partitioning ratios were determined directly from observed primary kinetic isotope effects. [α-^{2}H]Propargylglycine displays k_H/k_D values of about 3 on inactivation half-times. [α-^{3}H]-Propargylglycine gives release of tritium to solvent nominally stoichiometric with inactivation but, on correction for the calculated tritium isotope discrimination, partition ratios of four and six turnovers per monomer inactivated are indicated for cystathionine γ-synthase and methionine γ-lyase, respectively. The inactivation stoichiometry, using [α-^{14}C]-propargylglycine, is four labels per tetramer of cystathionine γ-synthase but usually only two labels per tetramer of methionine γ-lyase (half-of-the-sites reactivity). Two-dimensional urea isoelectrofocusing/NaDodSO$_4$ electrophoresis suggests (1) that both native enzymes are $\alpha_2\beta_2$ tetramers where the subunits are distinguishable by charge but not by size and (2) that, while each subunit of a cystathionine γ-synthase tetramer becomes modified by propargylglycine, only one α and one β subunit may be labeled in an inactive $\alpha_2\beta_2$ tetramer of methionine γ-lyase. Steady-state spectroscopic analyses during inactivation indicated that modified cystathionine γ-synthase may reprotonate C_2 of the enzyme–inactivator adduct, so that the cofactor is still in the pyridoxaldimine oxidation state. Fully inactivated methionine γ-lyase has λ_{max} values at 460 and 495 nm, which may represent conjugated pyridoximine paraquinoid that does not reprotonate at C_2 of the bound adduct. Either species could arise from Michael-type addition of an enzymic nucleophile to an electrophilic 3,4-allenic paraquinoid intermediate, generated initially by propargylic rearrangement upon a 4,5-acetylenic pyridoximine structure, as originally proposed for propargylglycine inactivation of γ-cystathionase [Abeles, R., & Walsh, C. (1973) *J. Am. Chem. Soc. 95*, 6124]. It is reasonable that cystathionine γ-synthase is the major in vivo target for this natural acetylenic toxin, the growth-inhibitory effects of which are reversed by methionine.

The acetylenic amino acid propargylglycine (2-amino-4-pentynoate, **1**) is a natural product elaborated by *Streptomycetes* (Scannel et al., 1971). Prior to its isolation as a metabolite from molds, the DL compound had been synthesized and found to be a growth inhibitor of certain strains of *Escherichia coli* and of yeast *Saccharomyces cerevisiae* (Gershon et al., 1949). Scannel et al. (1971) reported the reversal of propargylglycine-induced growth inhibition of *Bacillus subtilis* upon addition of L-methionine to the culture; thus, it seems probable that the major effect of this antibiotic in vivo is the inhibition of microbial methionine biosynthesis.

We have reasoned that pyridoxal phosphate dependent enzymes which generate β-carbanion (C_3) equivalents during catalysis might effect a propargylic rearrangement of the 4,5-acetylene to the electrophilic conjugated allenic PLP quinoid (**2**), which could function, in turn, as an active-site alkylating agent. This expectation has been borne out with studies on γ-cystathionase from rat liver (Abeles & Walsh,

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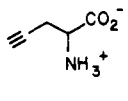

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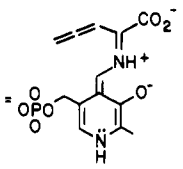

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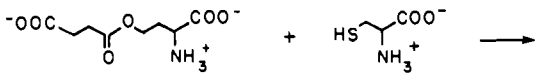
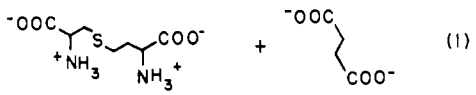
1973; Washtien & Abeles, 1977) and in preliminary experiments on pig heart L-alanine transaminase and on *Salmonella typhimurium* cystathionine γ-synthase (Marcotte & Walsh, 1975). Subsequently, the flavoprotein D-amino-acid oxidase has been shown to process D-propargylglycine for turnover as well as to suffer alkylation by it; these events have been investigated in detail (Horiike et al., 1975; Marcotte & Walsh, 1976, 1978a). The structures of the accumulating oxidation products have recently been elucidated (Marcotte & Walsh, 1978b).

In this paper we report on the inactivation of two pyridoxal P enzymes of methionine metabolism. The inactivation of one of these, cystathionine γ-synthase from *S. typhimurium* (and from other bacteria and plants), is the most likely in vivo mechanism for the antibiotic action of propargylglycine. Cystathionine γ-synthase carries out reaction 1, a key step in

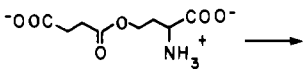


O-Succinyl-L-homoserine L-Cysteine

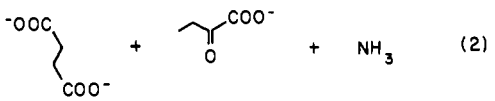


Cystathionine Succinate (1)

methionine biosynthesis which is a γ-replacement of the *O*-succinyl group of *O*-succinyl-L-homoserine by the thiolate anion of cysteine. In the absence of cysteine, cystathionine γ-synthase will catalyze γ-elimination alone, to give α-ketobutyrate, succinate, and ammonia, as shown in reaction 2.

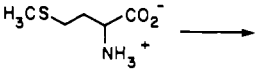


O-Succinyl-L-homoserine



Succinate α-Ketobutyrate (2)

In bacterial methionine degradation, an inducible methionine γ-lyase from *Pseudomonas ovalis* (Tanaka et al., 1977) catalyzes γ-elimination of methanethiol from L-methionine with concomitant production of α-ketobutyrate and ammonia, as shown in reaction 3.



Methionine

α-Ketobutyrate Methanethiol Ammonia (3)

These two enzymes are compared with regard to the kinetics of propargylglycine inactivation, the stoichiometry of active-site labeling, the susceptibility to reactivation, the electrophoretic mobility of the modified proteins, and the nature of the UV–visible chromophores generated during the course of inactivation.

Materials and Methods

Enzyme Purifications. Cystathionine γ-synthase (EC 4.2.99.9) was purified from *S. typhimurium* me-A (ATCC 25241) as described previously (Guggenheim & Flavin, 1969a; Johnston et al., 1979). The enzyme was homogeneous to polyacrylamide gel electrophoresis and to isoelectrofocusing. The specific activity for γ-elimination (reaction 2; assay described below) on OSHS[1] was observed to be 20 units/mg. The holoenzyme has two absorbance maxima at pH 7.3, one at 280 nm and one at 422 nm; the A_{280}/A_{422} is 3.90 for purified enzyme.

Methionine γ-lyase (EC 4.4.1.11) was purified from *P. ovalis* as described by Tanaka et al. (1977). The enzyme was homogeneous to polyacrylamide gel electrophoresis. The specific activity for γ-elimination (reaction 3; assay described below) on L-methionine was 3.5 units/mg. In our hands, methionine γ-lyase is somewhat unstable to storage either frozen or at 4 °C for long periods (6 months or more); as much as 20% variation in the specific activity is observed from one sample of enzyme to another. All experiments reported herein, therefore, have been appropriately controlled for variation in the specific activity of the enzyme.

Methionine γ-lyase has two absorbance maxima at pH 7.3, one at 280 nm and one at 418 nm; A_{280}/A_{418} is 3.90 for freshly purified enzyme. Preparations of the protein stored for long periods (or repeatedly frozen and thawed) exhibit increasing ratios of A_{280}/A_{418}; values as high as 4.7 have been obtained. It is possible that the enzyme loses pyridoxal phosphate on storage. We have not been successful in reversing either the loss of specific activity or the increase in the A_{280}/A_{418} ratio by dialysis of the enzyme against buffers containing pyridoxal 5-phosphate.

Preparation of Substrates and Inhibitors. *O*-Succinyl-L-homoserine (OSHS) was either prepared according to Flavin (1971) or purchased from Sigma Chemical Co. L-Methionine and L-cysteine hydrochloride were obtained from Sigma.

L-Propargylglycine (2-amino-4-pentynoate) and its radiolabeled derivatives were prepared according to the methods of Abeles & Walsh (1973). [2-^{14}C]-DL-Propargylglycine was prepared by using [^{14}C]diethylacetamidomalonate (New England Nuclear) as the starting material in the standard synthesis; the specific activity obtained was 500 cpm/nmol. [α-^{2}H]- and [α-^{3}H]-DL-propargylglycine were prepared by hydrolysis of propargyl diethylacetamidomalonate in $^{2}H_2O$ and $^{3}H_2O$ (1 Ci/mL), respectively. The specific activity of the α-tritiopropargylglycine obtained was 1460 cpm/nmol.

Kinetic Assays. Each of the two enzymes was assayed for the ability to form α-ketobutyrate (reactions 2 and 3) by the continuous reduction of the keto acid product in the presence of lactate dehydrogenase (Boehringer-Mannheim; from rabbit skeletal muscle) and NADH (Sigma). A typical 1.0-mL assay for cystathionine γ-synthase contained 50 mM KPP_i buffer, pH 8.3, at 37 °C, 5 mM OSHS,[2] 0.3 mg of LDH, and 2.0 μg of enzyme. The reaction conditions for the assay of methionine γ-lyase were identical except that a standard 1.0-mL reaction contained 4.0 μg of enzyme and was 25 mM in L-methionine.[3]

[1] Abbreviations used: OSHS, *O*-succinyl-L-homoserine; NADH, nicotinamide adenine dinucleotide, reduced form; KP_i, potassium inorganic phosphate; KPP_i, potassium inorganic pyrophosphate; LDH, lactate dehydrogenase; PLP, pyridoxal 5-phosphate; $NaDodSO_4$ (SDS in the figures), sodium dodecyl sulfate.

[2] The K_M for OSHS is 0.18 mM (Johnston et al., 1979).

The rate of product formation was monitored as a function of the disappearance of NADH absorbance at 340 nm.

Reactions with Propargylglycine. (*a*) *Kinetics of Inactivation.* The following general protocol was employed for the determination of the rate of enzymatic inactivation. At time zero, enzyme was added to a solution containing an appropriate concentration of propargylglycine in 50 mM KPP_i buffer, pH 8.3, at 37 °C. Aliquots (usually 10 μL or less) were removed at intervals and assayed for remaining enzymatic activity by dilution to a 1.0-mL solution of the appropriate standard assay mixture (see above). The half-time for inactivation was obtained from a semilog plot of percent activity remaining vs. time.

For inactivation of cystathionine γ-synthase, the inactivation system usually contained 20 μg of enzyme in 100-μL total volume. For inactivation of methionine γ-lyase, the system usually contained 80 μg of enzyme in 250-μL total volume. In separate experiments, the propargylglycine concentrations were varied (0.02–0.50 mM) so as to determine values of K_I and k_2 for the process

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI \xrightarrow{k_2} E\text{–}I \qquad (4)$$

(*b*) *Estimation of the ^2H Isotope Effect on Inactivation.* The primary deuterium isotope effect on the half-time of inactivation was determined for both enzymes by using [α-^{2}H]-DL-propargylglycine in the standard inactivation assay described above. For the inactivation of cystathionine γ-synthase, the concentration of α-deuteriopropargylglycine was 0.1 mM; for the inactivation of methionine γ-lyase, the α-deuteriopropargylglycine concentration used was 0.08 mM. Reaction half-times for inactivation by 0.1 and 0.08 mM [α-^{1}H]-DL-propargylglycine were obtained for each enzyme, respectively. The primary deuterium isotope effect is thus given as $t_{1/2(D)}/t_{1/2(H)}$.

(*c*) *Tritium "Washout" from [α-^3H]Propargylglycine.* The ability of each enzyme to catalyze α-proton exchange between propargylglycine and solvent water was determined in the following way. A 500-μL solution was prepared which was 0.1 mM [α-^{3}H]-DL-propargylglycine and enzyme (0.19 mg of cystathionine γ-synthase or 0.16 mg of methionine γ-lyase) and 50 mM KPP_i buffer, pH 8.3, at 37 °C. The inactivation was initiated by addition of propargylglycine; at timed intervals 50-μL aliquots were removed and loaded onto Dowex 50 H^+ columns. The columns were eluted with 2.0 mL of water, and the water was counted in 15.0 mL of ACS scintillant. Small aliquots (5 μL in the case of the cystathionine γ-synthase reaction and 25 μL in the case of the methionine γ-lyase reaction) were similarly withdrawn at intervals and were assayed for residual enzymatic activity by dilution to the standard ketobutyrate-forming reactions. This procedure allowed the simultaneous estimation of the rates of both inactivation and of tritium "washout to solvent" from α-tritiopropargylglycine.

(*d*) *Thiol Reactivation.* Each of the two enzymes was inactivated as described above; once the residual activity had fallen to zero, the inactivation system was made 10 mM in one of the following thiols: dithiothreitol, 2-mercaptoethanol, ethanethiol, butanethiol, or benzenethiol. The mixtures were then assayed for the recovery of catalytic activity by dilution of 20-μL aliquots of the enzyme–propargylglycine–thiol incubation to the appropriate ketobutyrate-forming assay.

Stoichiometry of Inactivation. The stoichiometry of inactivation was determined by the following procedure. Each enzyme (1.8 mg of cystathionine γ-synthase and 0.95 mg of methionine γ-lyase) was reacted in 1.0 mL with 0.2 mM [α-^{14}C]-DL-propargylglycine (500 cpm/nmol); inactivation was monitored in the standard way by dilution to a ketobutyrate-forming assay. Inactivation was allowed to proceed until residual activity had fallen to zero; usually 120 min was required for full inactivation of cystathionine γ-synthase and 45 min was required for methionine γ-lyase. The protein-inactivator solution was then loaded onto a Sephadex G-25 column (1 × 33 cm; 25.9 mL) which had been previously calibrated for separation of bovine serum albumin (1.0 mg) and 0.19 mM [^{14}C]proline (New England Nuclear; 270 mCi/mmol). The column was eluted (6 mL/h) with 10 mM KP_i buffer, pH 7.3, at 4 °C, and 0.5-mL fractions were collected. Each fraction was examined for absorbance at 280 nm and counted for ^{14}C radioactivity.

Spectral Studies. Steady-state absorbance spectra for the reactions of cystathionine γ-synthase and methionine γ-lyase with propargylglycine were obtained by using an Hitachi Perkin-Elmer 200 scanning spectrophotometer. A typical spectral analysis involved the following protocol. An initial absorbance scan was made on a solution of enzyme (usually about 0.5 mg) in 480 μL of KPP_i buffer, pH 8.3, at 20 °C. At time zero, 20 μL of 1.0 mM L-propargylglycine was added to bring the final reaction to 50 mM KPP_i, pH 8.3, 0.04 mM propargylglycine, and approximately 1.0 mg/mL enzyme. Absorbance scans were made at timed intervals following the addition of inactivator; aliquots were simultaneously assayed to correlate the spectral events with the loss of catalytic activity.

Electrophoresis and Electrofocusing. Disc gel polyacrylamide electrophoresis was performed on both active and inactivated enzymes by using the methods of Ornstein (1964); sodium dodecyl sulfate–polyacrylamide gel analyses were performed according to the procedures of Weber & Osborne (1969).

Each enzyme (both native and propargylglycine modified) was subjected to two-dimensional urea isoelectrofocusing/$NaDodSO_4$–poylacrylamide gel electrophoresis, according to the procedures of O'Farrell (1975). This technique consists of isoelectrofocusing in 8.0 M urea across a pH gradient from 3.0 to 7.0, which separates the dissociated subunits of an oligomer according to differences in their isoelectric points. The electrofocusing gel is then adhered to a $NaDodSO_4$–polyacrylamide slab gel, and electrophoresis is carried out at a 90° angle from the dimension of the isoelectrofocusing. The focused peptides are thus further resolved, but in the second dimension according to differences in their molecular sizes.

Results

Kinetic Profiles of Inactivation. L-Propargylglycine is a time-dependent inactivator of both cystathionine γ-synthase and methionine γ-lyase. Typical semilog plots of remaining enzymatic activity vs. time are given in Figure 1. The pertinent kinetic data for inactivation of both enzymes are given in Table I; the kinetic parameters for γ-elimination (reactions 2 and 3) are also included in Table I.

The kinetics of inactivation, as shown in Figure 1, are notably and reproducibly biphasic. The initial first-order rate (k_2 of eq 4) is more than twofold greater for methionine γ-lyase than for cystathionine γ-synthase (Table I). A break in the initial phase of inactivation occurs between 10 and 20% residual activity for the examples given in Figure 1; the level of residual enzymatic activity at which the break in the initial inactivation rate curve occurs is dependent upon the concentration of inactivator, below saturation values (data not

[3] The K_M for methionine is 2.3 mM (see Table I).

Table I: Kinetic Data for the Reactions of Cystathionine γ-Synthase and Methionine γ-Lyase

compd	cystathionine γ-synthase				methionine γ-lyase			
	K_M (mM)	V_{max} (units/mg)	K_I (mM)	k_2 (s^{-1} × 10^3)	K_M (mM)	V_{max} (units/mg)	K_I (mM)	k_2 (s^{-1} × 10^3)
O-succinyl-L-homoserine	0.18[a]	21.0[a]						
L-methionine					2.3	3.2		
L-propargylglycine			0.13[b]	15.0[b]			0.20[b]	37.0[b]
			0.08[c]	2.2[c]			0.07[c]	5.5[c]

[a] Data from Johnston et al. (1979). [b] Data are for the initial pseudo-first-order rate of inactivation. [c] Data are for the second pseudo-first-order rate of inactivation.

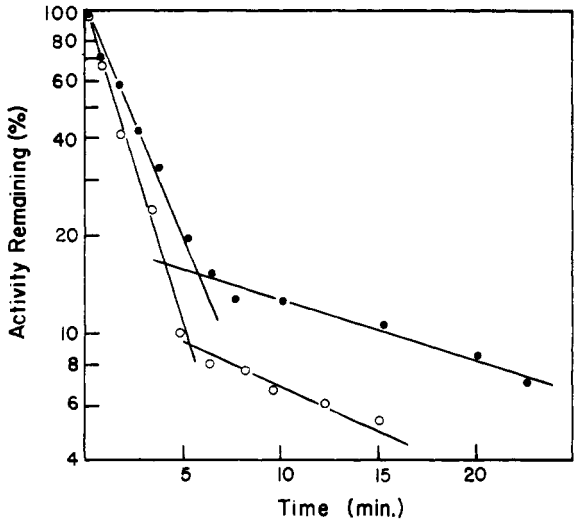


FIGURE 1: Kinetics of inactivation of cystathionine γ-synthase (●) and of methionine γ-lyase (○) by 0.1 mM L-propargylglycine. (Reaction conditions are described in the text.)

shown). The second, or slower, phase of first-order inactivation proceeds until the enzyme becomes fully inactivated.

The second first-order rate (k_2') for methionine γ-lyase is again more than double that obtained for cystathionine γ-synthase (Table I). The binding constants for the two slower phases (K_I') are significantly lower than those obtained for each of the two faster, initial phases (K_I).

We have attempted to demonstrate substrate protection against propargylglycine inactivation of cystathionine γ-synthase by using the cosubstrate (for reaction 1) cysteine.[4] At cysteine concentrations (0.1 mM) which approximate the K_M value for cysteine in reaction 1 (0.23 mM; Johnston et al., 1979), the cosubstrate does not offer protection against 0.1 mM propargylglycine inactivation. In fact, the half-time for inactivation in the presence of cysteine ($t_{1/2}$ = 2.0 min) is slightly less than in its absence ($t_{1/2}$ = 2.5 min). At high cysteine concentration (10 mM), the rate of propargylglycine (0.1 mM) inactivation is accelerated ($t_{1/2}$ = 1.0 min). Moreover, cysteine alone gave time-dependent irreversible inactivation of cystathionine γ-synthase with pseudo-first-order kinetics. The value for the first-order rate constant (k_2) is 1.1 × 10^{-3} s^{-1}, and the K_I for cysteine is 0.44 mM, twice the K_M value for cysteine in γ-replacement previously reported by Johnston et al. (1979). Thus, cysteine and propargylglycine appear to inactivate the enzyme by independent mechanisms.

Turnover of Propargylglycine. Partition Ratios. (a) Kinetic Evidence for Turnover. Neither cystathionine γ-synthase nor methionine γ-lyase suffers complete inactivation by stoichiometric concentrations of propargylglycine, even in experiments where equimolar concentrations of enzyme and inactivator are well above the K_I value of propargylglycine for the specific enzyme undergoing inactivation. Figure 2 shows that the extent of inactivation obtained for each enzyme is a reasonably linear function of the inactivator/propargylglycine concentration ratio. Inactivation in excess of 95% is obtained for cystathionine γ-synthase with a minimum molar ratio of propargylglycine to enzyme monomer of 4:1; the corresponding value for complete inactivation of methionine γ-lyase is approximately 8:1 per monomer equivalent (Table II).

These values alone suggest that both enzymes may process propargylglycine for turnover as well as for inactivation. Insofar as turnover is accomplished, each of these concentration ratios establishes an absolute upper limit upon the respective partition ratios for turnover to inactivation. That is, an active monomer of cystathionine γ-synthase may be expected to process to some product up to, but no more than, 4 equiv of propargylglycine for every inactivation event. Similarly, methionine γ-lyase may turn over an average of eight propargylglycine molecules to some product for every molecule of enzyme monomer inactivated.

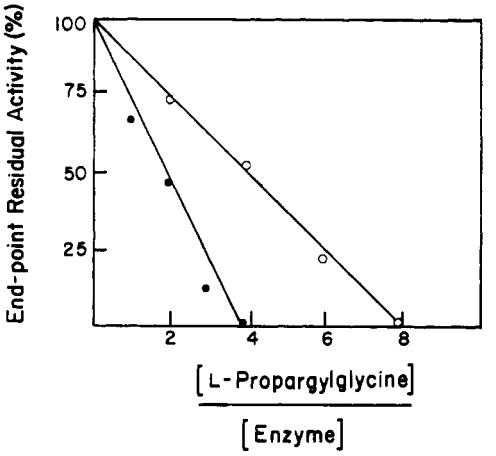


FIGURE 2: Inactivation of cystathionine γ-synthetase (●) and methionine γ-lyase (○) observed as a function of the concentration ratio of L-propargylglycine to enzyme.

Table II: Kinetic Data for the Enzymatic Processing of Propargylglycine

enzyme	stoichiometry of ^3H release vs. inactivation[a]	max partition ratio[b]	isotope effects	
			k_H/k_D	k_H/k_T
cystathionine γ-synthase	0.82	4	2.5	3.75
methionine γ-lyase	1.02	8	3.25	5.45

[a] Per enzyme monomer. [b] Predicted from the data of Figure 3.

[4] The use of OSHS in substrate-protection experiments is prevented in that this is the consumed substrate for the standard activity assay (reaction 2).

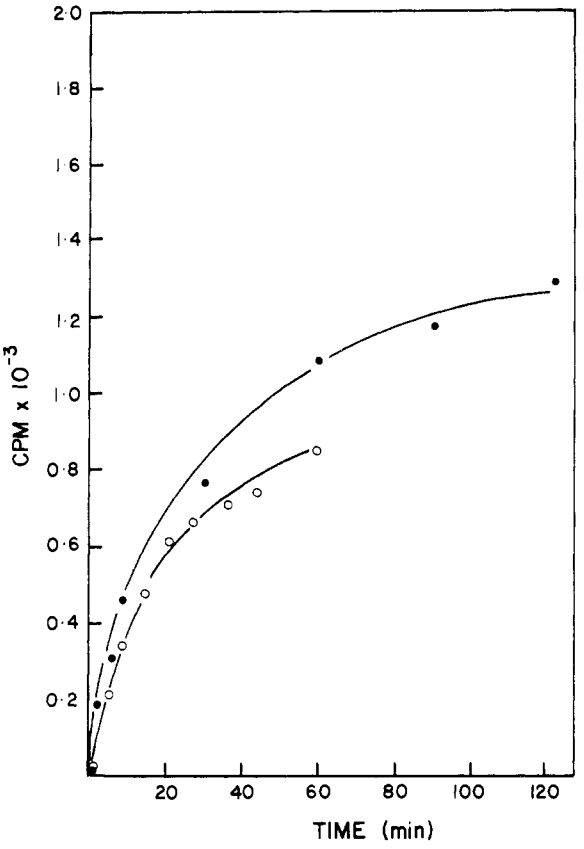


FIGURE 3: Rates of tritium release from α-[^{3}H]-DL-propargylglycine catalyzed by cystathionine γ-synthase (●) and by methionine γ-lyase (○). (Reaction conditions are described in the text.)

(*b*) *Tritium Washout to Solvent.* We have attempted to detect the processing of propargylglycine by reaction of each enzyme with specifically labeled [α-^{3}H]-DL-propargylglycine, according to the following rationale. If Propargylglycine is both active-site-directed and mechanistically based in its mode of action, α-proton abstraction is required as a first step in alkylation and must be strictly antecedent to it (see Scheme I). Thus, if every proton abstraction leads to inactivation (if killing is 100% efficient), then every inactivation event effected by α-tritiopropargylglycine will give a stoichiometric amount of tritium "washed out" to solvent. (The critical assumption here is that proton abstraction proceeds without a detectable primary kinetic isotope effect.[5]) On the other hand, if the enzyme partitions between turnover and inactivation, the stoichiometry of solvent tritium produced vs. inactivation will exceed unity.

The data for the rate of tritium washed out to solvent water from α-tritiopropargylglycine by cystathionine γ-synthase and by methionine γ-lyase are shown in Figure 3. Based on a specific activity for labeled propargylglycine of 1460 cpm/nmol, the expected recovery of radioactivity (stoichiometric inactivation, no isotope effect) is 3475 cpm for cystathionine γ-synthase. The total counts recovered after 120 min of incubation (at which time the enzyme had less than 10% its original activity) was 2850 cpm (corrected for 42% efficiency in tritium counting), which corresponds to 0.82 mol of α-tritium released per mol of enzyme monomer inactivated (Table II).

[5] A secondary, but no less complicating, requirement is that the enzymic base which captures the α-proton of propargylglycine must not be sequestered from bulk solvent, so that tritium may fully equilibrate with solvent water.

For methionine γ-lyase, the total radioactivity recovered after 30 min (less than 10% residual activity) was 3000 cpm (corrected); that expected for 1:1 stoichiometry is 5860 cpm. The ratio in this case is 0.51 mol of tritium released per mol of enzyme inactivated. However, since we have observed that the stoichiometry of label incorporation into methionine γ-lyase is usually 0.5 label/enzyme monomer (vida infra), the predicted recovery of radioactivity should be only 2930 cpm. And thus, the actual ratio of tritium release per monomer inactivated is a stoichiometric 1.02 (Table II).

These data suggest that propargylglycine inactivation is 100% efficient and, as such, are at apparent odds with the results given in Figure 2 above, which are consistent with some processing of the inactivator. However, one might conclude that inactivation is less than completely efficient if the inactivation proceeds with isotope discrimination against tritium. In fact, if partitioning between turnover and inactivation does occur, an apparent 1:1 stoichiometry of tritium release and inactivation can obtain only if the primary tritium isotope effect upon the rate of inactivation equals the partition ratio (i.e., $k_H/k_T \simeq 4$ for cystathionine γ-synthase and $k_H/k_T \simeq 8$ for methionine γ-lyase).

It might be noted in passing that the tritium release data confirm that the reaction of each enzyme and the inactivator is a catalytic process and thus qualifies propargylglycine as a "suicide substrate".

(*c*) *Kinetic Isotope Effects.* We have determined the primary deuterium isotope effect on the half-time for inactivation of both enzymes; these data are given in Table II. The respective values for the primary tritium isotope effect have been calculated according to the Swain relationship (Swain et al., 1958):

$$\log (k_H/k_T) = 1.44 \log (k_H/k_D) \qquad (5)$$

and these values are also given in Table II. Note that the values for the tritium isotope effects (k_H/k_T = 3.75 and 5.5, respectively), which must express the maximum number of α-protons abstracted from propargylglycine for each inactivation event ($k_H/k_T \equiv$ partition ratio), agree well with the maximal predicted values (four and eight turnovers per inactivation for each enzyme, respectively) from inactivation titration data.

Thiol Reactivation. Four of five thiols tested are variously effective for the partial reactivation of fully inactivated methionine γ-lyase. In order of decreasing efficacy, the reactivating thiols are dithiothreitol (30%), ethanethiol (25%), butanethiol (20%), and benzenethiol (18%). Mercaptoethanol does not at all reactivate the enzyme.

Cystathionine γ-synthase, by contrast, is essentially resistant to thiol reactivation. Dithiothreitol (10 mM) gives variable but only slight reactivation; the maximum recovery is never greater than 5% activity. None of the other thiols effect any detectable reactivation of the enzyme.

Stoichiometry of Labeling. Figure 4 presents the elution profiles of the two enzymes fully inactivated by [^{14}C]-propargylglycine and subsequently exposed to Sephdex G-25 gel filtration. A peak of radioactivity is seen to coelute in each case with the protein absorbance at 280 nm, a finding which confirms our expectation that the inactivation is covalent and irreversible.

The total radioactivity recovered under the protein peak in Figure 4A corresponds to a stoichiometry of labeling for cystathionine γ-synthase of 3.88 mol of propargylglycine per mol of enzyme tetramer (or, one label per monomer). The pyridoxal content of the enzyme (a tetramer of four putatively identical subunits) has been determined previously (Kaplan

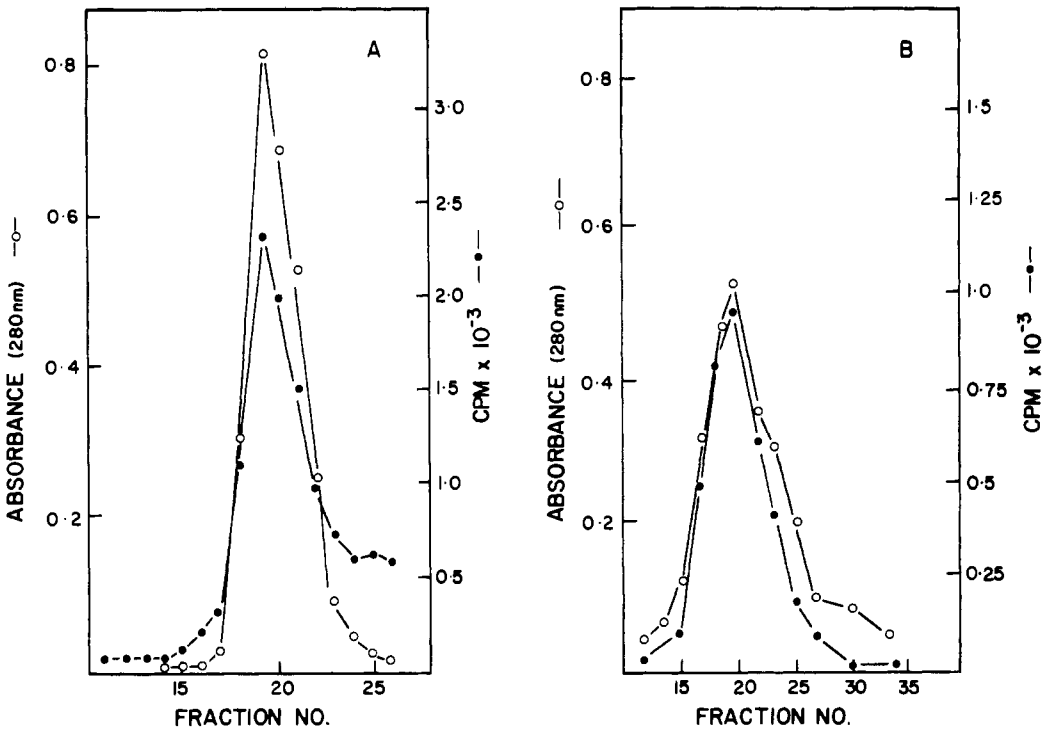


FIGURE 4: Sephadex G-25 elution profiles for cystathionine γ-synthase (A) and methionine γ-lyase (B) following inactivation by α-[^{14}C]-DL-propargylglycine. (Reaction conditions are described in the text.)

& Flavin, 1966) to be one PLP per subunit.

The total radioactivity recovered under the protein peak in Figure 4B corresponds to a stoichiometry of labeling for methionine γ-lyase of 2.0 mol of propargylglycine per mol of enzyme tetramer (or, one label per dimer). The enzyme is a tetramer assumed to be composed of four identical subunits[6] (but see below), and we have determined [by the procedures of Wada & Snell (1961)] that each monomer contains one molecule of PLP. Thus, unlike cystathionine γ-synthase, methionine γ-lyase shows "half-of-the-sites reactivity" (Levitzki et al., 1971) in propargylglycine-induced inactivation.

Both enzymes lack detectable catalytic activity against their respective substrates after elution from the gel filtration column, suggesting that the label is stable to solvolysis. Methionine γ-lyase remains inactive even after dialysis (for 24 h) of the modified protein against KP_i buffer, pH 7.3, containing pyridoxal phosphate (50 mM). The modified cystathionine γ-synthase may experience slow solvolysis of the covalent label in that dialysis (for 24 h) gives 16% recovery of the catalytic activity; this recovery is obtained even when the dialysis buffer lacks added pyridoxal phosphate.

Certain preparations of methionine γ-lyase required considerably longer incubation periods to achieve complete inactivation than the standard 45 min employed in the radiolabeling experiments described above. The reason for this variation is unclear. The data for one such experiment, wherein enzyme inactivated by [^{14}C]propargylglycine (60 cpm/nmol) was subjected to Sephadex G-25 gel filtration at various times during the course of inactivation, are given in Figure 5. Note from the figure that there is an initially rapid loss of catalytic activity, to less than 20% within 10 min. Then follows a loss of activity at a much slower rate such that up to 8 h are required before the enzyme is fully inactivated.

The data of Figure 5 also show that the rate of ^{14}C-labeling is synchronized with the sigmoidal loss of catalytic activity. That is, within 1 h, after which time the enzyme shows less than 10% residual activity, 2.5 mol of [^{14}C]propargylglycine has become incorporated per mol of enzyme tetramer. During the subsequent 7-h incubation, while catalytic activity drops from a residual 10% to zero, an additional 1.5 mol of [^{14}C]propargylglycine is incorporated into the enzyme. Thus, the final stoichiometry in this experiment is four labels per tetramer (or one label per monomer), although the first two labels are introduced much faster (and their introduction parallels the loss of the bulk of the catalytic activity) than are the last two labels.

Electrophoresis and Electrofocusing of the Modified Proteins. Both cystathionine γ-synthase and methionine γ-lyase show single bands of protein on polyacrylamide gels; the respective R_f values are 0.31 and 0.42 toward the cathode. After complete inactivation by propargylglycine, each protein continues to run as a single band on polyacrylamide gels, but in both cases the modified enzyme is more anionic. Inactivated cystathionine γ-synthase moves with an R_f of 0.36; the corresponding value is 0.50 for modified methionine γ-lyase. On the basis of these observations alone, the electrophoretic data tend to suggest that every individual enzyme tetramer has been identically modified, and modified methionine γ-lyase is migrating as a tetramer still.

Both enzymes migrate as single bands on $NaDodSO_4$–polyacrylamide gels. There are no changes in the R_f values before and after inactivation for either enzyme, consistent with the expectation that propargylglycine alkylation does not affect the detectable molecular size of the protein monomers.

The two-dimensional urea electrofocusing/$NaDodSO_4$ electrophoresis gel patterns for native forms of both enzymes are shown in Figure 6. Note that there are two spots of protein staining in each gel, of R_f values in the electrofocusing dimension of 0.40 and 0.45 (α and β of Figure 6A) for

[6] Soda and his colleagues originally reported (Tanaka et al., 1977) that the enzyme is made up of two nonidentical subunits of 48 000 and 44 000 molecular weight, respectively. But it is likely that the heterogeneity observed resulted from proteolysis of a single 48 000 molecular weight subunit (Soda, unpublished experiments). The enzyme preparations used in this study are homogeneous tetramers of four subunits identical *at least as to molecular size.*

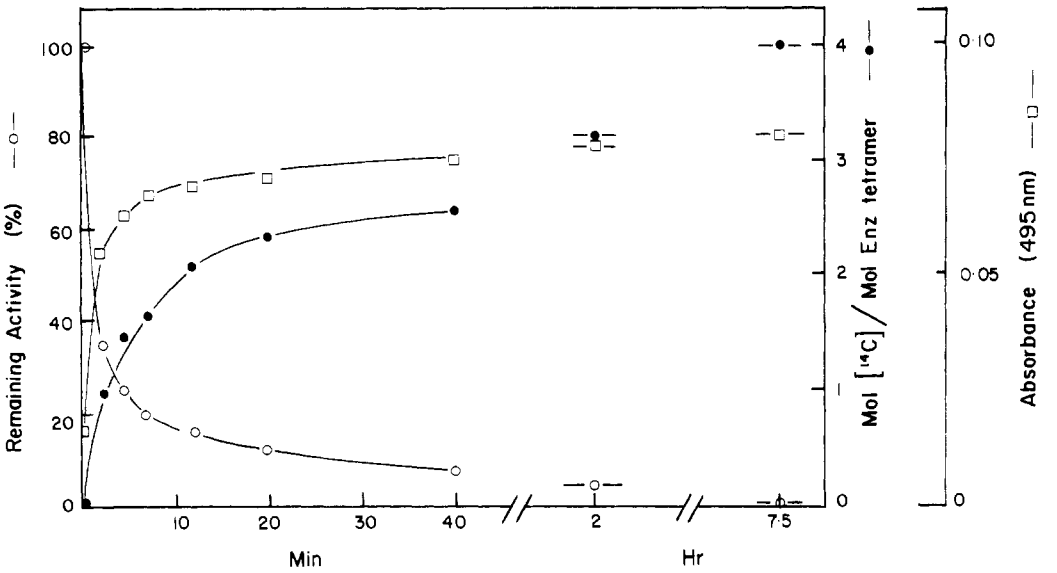


FIGURE 5: Inactivation of methionine γ-lyase (about 0.5 mg) by 2.0 mM [^{14}C]propargylglycine (60 cpm/nmol). Multiple inactivation experiments were followed with time and monitored for loss of catalytic activity (○), for absorbance at 495 nm (□), and for the incorporation of radiolabel (●). The stoichiometry data were obtained by gel filtration of the enzyme–propargylglycine mixture on Sephadex G-25 columns, as described in the text.

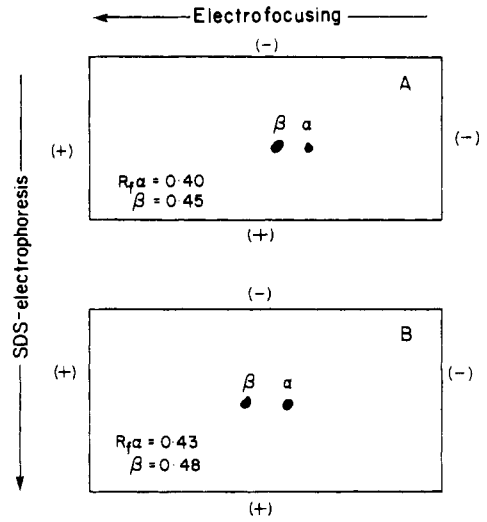


FIGURE 6: Diagrammatic representations of the two-dimensional urea isoelectrofocusing/NaDodSO$_4$ electrophoresis gel patterns for native cystathionine γ-synthase (A) and for native methionine γ-lyase (B). Isoelectrofocusing was performed across a pH gradient from 3.0 to 7.0. R_f values listed are for migrations in the electrofocusing dimension.

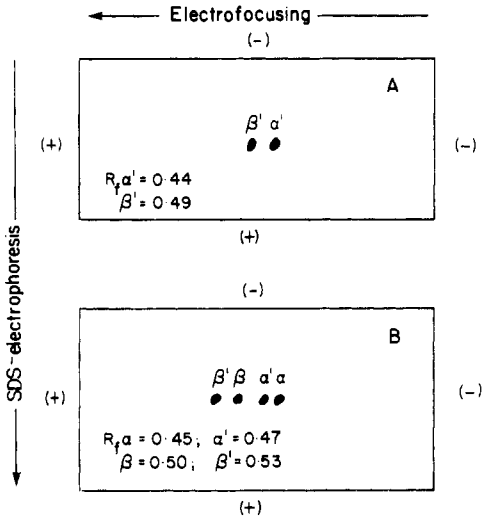


FIGURE 7: Diagrammatic representations of the two-dimensional urea isoelectrofocusing/NaDodSO$_4$ electrophoresis gel patterns for cystathionine γ-synthase (A) and for methionine γ-lyase (B) fully inactivated by L-propargylglycine. Isoelectrofocusing was performed across a pH gradient from 3.0 to 7.0. R_f values listed are for migrations in the electrofocusing dimension.

cystathionine γ-synthase and of R_f 0.43 and 0.48 (α and β of Figure 6B) for methionine γ-lyase. These patterns demonstrate that both enzymes exhibit subunit heterogeneity in the isoelectrofocusing dimension. Note that each pair of spots is on a horizontal (R_f values in the vertical dimension are 0.45 for Figure 6A and 0.52 for Figure 6B); that is, each enzyme displays subunit homogeneity in the NaDodSO$_4$ electrophoresis dimension. Thus, we may conclude that both enzyme oligomers are composed of subunits which are essentially identical as to molecular size and nonidentical as to molecular charge. The intensity of staining suggested that the different subunit populations are relatively equal.

Figure 7 presents the results of the two-dimensional gels for both enzymes which have been completely inactivated by propargylglycine. Note for cystathionine γ-synthase (Figure 7A) that the gel pattern is like that observed for native enzyme (Figure 6A) but that each spot is relatively more anionic in the isoelectrofocusing dimension than that of untreated enzyme; the respective R_f values are now $\alpha' = 0.44$ and $\beta' = 0.49$. This observation is in accord with the results obtained from polyacrylamide electrophoresis, which suggest that the enzyme tetramer has a higher (more anionic) pI value after modification by propargylglycine. The distinct pairs of R_f values for α and β (Figure 6A) and α′ and β′ (Figure 7A) would seem to suggest that both types of monomer become labeled, and this result is consistent with a stoichiometry of inactivation for cystathionine γ-synthase of one label per monomer.

The corresponding results for methionine γ-lyase completely inactivated by propargylglycine (Figure 7B) are notable in that four spots of protein are distinct in the isoelectrofocusing dimension and nondistinct in the NaDodSO$_4$ electrophoresis dimension. Thus, it would appear that fully inactivated enzyme is composed of four types of monomer (α, α′, β, β′), each identical as to molecular size and each nonidentical as to molecular charge. Two of the subunits (α and β) have R_f values (0.45 and 0.50) which closely correspond to those obtained for the subunits of Figure 6B and may represent

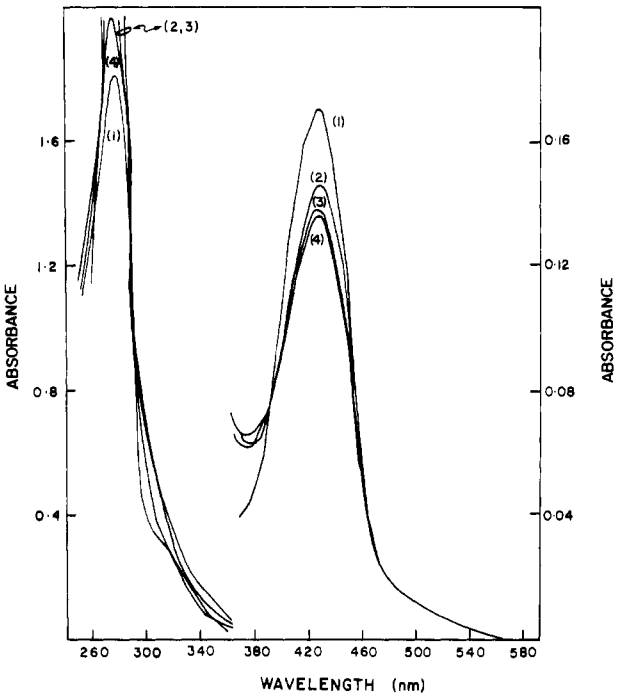


FIGURE 8: Steady-state spectra of cystathionine γ-synthase during inactivation by L-propargylglycine. (Reaction conditions are described in the text.) Curve 1 was obtained prior to addition of the inactivator; curves 2–4 were obtained 10, 30, and 120 min, respectively, after the addition of propargylglycine.

unlabeled nonidentical subunits. The other pair of spots (α' and β') has R_f values (0.47 and 0.53) which suggest that the native α and β spots have "split", giving new subunits with relatively higher (more anionic) p*I* values; these may represent two nonidentical *labeled* subunits. This pattern, where one α and one β subunit of an $\alpha_2\beta_2$ tetramer become alkylated by propargylglycine, is consistent with a stoichiometry of inactivation for methionine γ-lyase of one label per dimer.[7]

Spectral Analysis. We have examined the steady-state UV–visible spectra during inactivation of both enzymes, and these results are given in Figures 8 and 9.

The inactivation of cystathionine γ-synthase (Figure 8) by propargylglycine proceeds with largely uncomplicated spectral patterns. Addition of inactivator to the fully active enzyme (curve 1) produces a progressive but modest loss of the pyridoxaldimine chromophore at 422 nm (curves 2–4). Fully inactivated enzyme (curve 4) has an absorbance at 422 nm which is 80% that given by the enzyme prior to addition of propargylglycine.[8] Figure 8 shows a large enhancement of the absorbance at 280 nm upon addition of propargylglycine. This absorbance is so intense that during the course of reaction the A_{280} is above the sensitivity of detection of the spectrophotometer. After 120 min (by which time the enzyme is fully inactivated), the 280-nm band appeared to give diminished absorbance (curve 4). It is not clear whether this spectral behavior arises from transient modification of the protein absorbance alone or from some cofactor–adduct chromophore with a high extinction coefficient in the ultraviolet.

The inactivation of methionine γ-lyase proceeds with the development of a complicated steady-state spectral pattern (Figure 9A). Addition of propargylglycine to the resting enzyme (curve 1) results in the decay of the 418-nm absorbance and in the progressive development of sharp bands of absorbance at 460 and 495 nm (curves 2–5). It seems likely that these two high-wavelength bands represent the accumulation of a single chromophore; their rates of accumulation are observed to be strictly identical. Note also, from the data presented in Figure 5, that the rate of accumulation of the 495-nm absorbance parallels the rate of loss of catalytic activity.

Fully inactivated methionine γ-lyase gives a spectrum composed nearly entirely of the two sharply defined bands at 460 and 495 nm (Figure 9B, curve 1). This final spectrum shows also a shoulder of absorbance at 435 nm; it is difficult, however, to determine if this band represents a distinct chromophore or if, rather, it represents the overlap of the 465-nm species and any residual 418-nm absorbance.

The chromophores which characterize fully inactivated methionine γ-lyase can be quenched completely by the addition of thiol, when the spectral solution is made 10 mM in dithiothreitol (Figure 9B, curve 2). The resulting spectrum is similar to, but not strictly isospectral with, fully active enzyme. There is an approximately 60% recovery of the pyridoxaldimine 418-nm absorbance and a residual shoulder of absorbance between 490 and 495 nm. This spectrum is stable to prolonged incubation (30–60 min) at 20 °C. The enzyme was assayed after the spectrum of Figure 9B, curve 2, was obtained and was seen to regain a maximum of 25% activity after 15 min of incubation with thiol.

Discussion

Kinetic Interpretation. Cystathionine γ-synthase and methionine γ-lyase both suffer time-dependent irreversible inactivation by the acetylenic amino acid propargylglycine; the rates of inactivation are linear in remaining enzyme. These are results which meet the generally held minimal kinetic criteria for active-site-directed, covalent enzyme inactivation (Walsh, 1978a; Abeles & Maycock, 1976), but we make here the somewhat unusual observation that the semilog plots of activity vs. time for both enzymes are biphasic.[9]

Biphasic kinetics have been reported for the inactivation of γ-cystathionase[10] by aminooxyacetate (Beeler & Churchich, 1976), a result attributed to the nonequivalent binding of the inactivator to the pyridoxal-containing sites of the oligomeric protein. We have similarly noted that distinct binding constants (Table I) are obtained for the "fast" and "slow" steps of the propargylglycine-induced inactivations. Biphasic reactivity which results from nonequivalent binding is conceivable, especially for oligomeric enzymes of the type examined here which show microheterogeneity of subunit composition.

Kinetic cooperativity between subunits is also likely to result in biphasic reactivity, such that rapid modification of one

[7] We have not yet characterized the two-dimensional gel pattern for enzyme which bears four labels per tetramer. But we predict that methionine γ-lyase so labeled will give a gel pattern of two spots (α' and β') similar to that obtained for fully inactivated cystathionine γ-synthase.

[8] Dilution of the chromophore by addition of the inactivator (10 μL in 1.0 mL) is 1.0%.

[9] Childs & Bardsley (1975) have argued that the usual proposal for pseudo-first-order kinetics may be an inappropriate model for describing the events involved in active-site-directed covalent labeling of enzymes. They suggest, in fact, that an otherwise catalytic inactivation need not generate linear semilogarithmic plots of residual enzymic activity vs. time. While we do not observe inactivation kinetics which violate the pseudo-first-order hypothesis, biphasic reactivity suggests that the kinetics of inactivation reactions—induced either by affinity labels or by suicide substrates—may be rather more complicated (and revealing?) than hitherto suspected.

[10] γ-Cystathionase is a tetramer which contains one pyridoxal phosphate per subunit (Churchich et al., 1975). The enzyme catalyzes cleavage of the thioether linkage of L-cystathionine to yield α-ketobutyrate, L-cysteine, and ammonium ion.

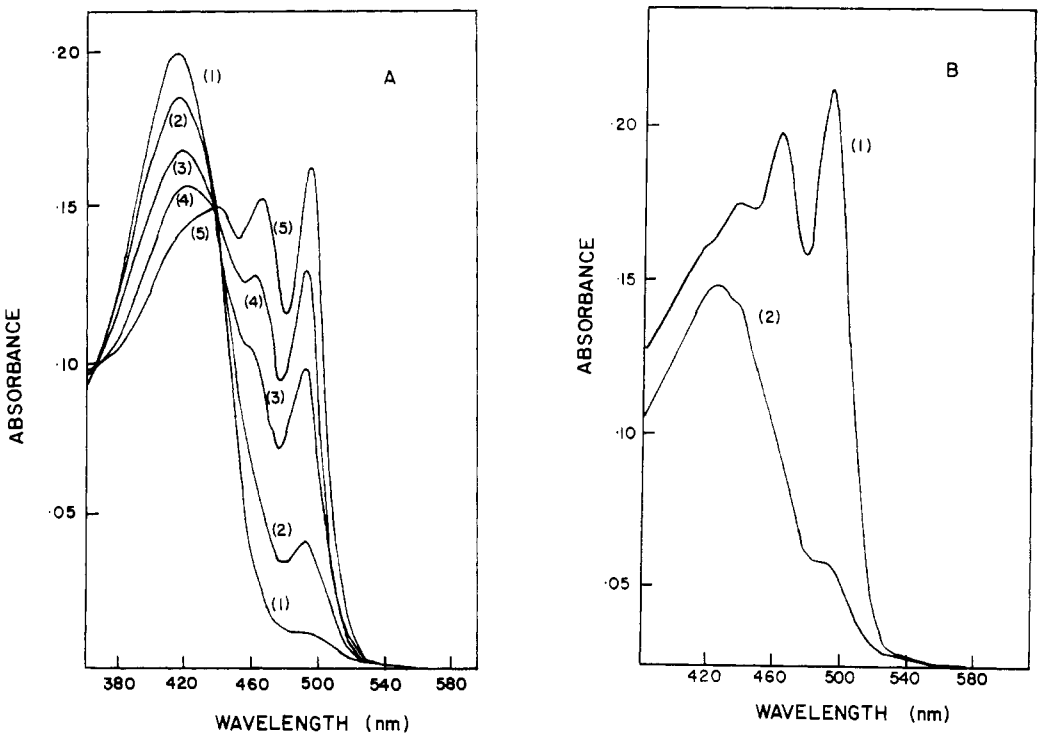


FIGURE 9: (A) Steady-state spectra of methionine γ-lyase obtained during inactivation by L-propargylglycine. (Reaction conditions are described in the text.) Curve 1 was obtained prior to the addition of the inactivator; curves 2–5 were obtained 2, 10, 45, and 60 min, respectively, after the addition of propargylglycine. (B) Spectral patterns obtained when fully inactivated methionine γ-lyase (curve 1) is made 10 mM in dithiothreitol (curve 2). (Reaction conditions are described in the text.)

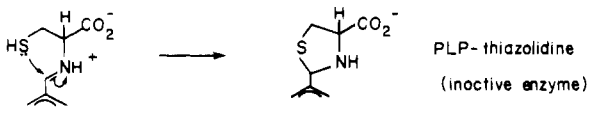
Table III: Protein Structural Data for Pyridoxal Enzymes Which Undergo Propargylglycine Inactivation

enzyme	native M_r	oligomeric structure	molecules of propargylglycine incorporated per inactivated tetramer	subunit composition	ref
cystathionine γ-synthase (*S. typhimurium*)	160 000	tetramer	4	$\alpha_2\beta_2$	this work
methionine γ-lyase (*P. ovalis*)	180 000	tetramer	2–4	$\alpha_2\beta_2$	this work
γ-cystathionase (rat liver)	160 000	tetramer	2[a]	ND[b]	Washtien & Abeles (1977)

[a] Enzyme is inactive to substrate but will react with another 2 equiv of trifluoroalanine. [b] ND = not determined.

subunit alters the ability of an adjacent subunit to experience alkylation at the same rate. This is an especially persuasive hypothesis for biphasic inactivation of methionine γ-lyase, which displays extreme negative cooperativity with regard to propargylglycine inactivation. Kinetic cooperativity is discussed further below.

Cysteine Inactivation. Cystathionine γ-synthase has been shown to catalyze α-proton exchange between solvent deuterium and both L-cystine and *S*-methyl-L-cysteine (Guggenheim & Flavin, 1969b). In light of these findings, it is clear that cysteine can undergo transaldimination with the enzyme; one mechanism for covalent inactivation, as suggested below,



might involve attack of the cysteinyl thiol upon the benzylic carbon of the amino acid–pyridoxaldimine adduct. The resulting pyridoxal thiazolidine would be an inactive enzyme. We have not attempted to verify this speculation.

Cystathionine γ-synthase gives ping pong bibi kinetics (Johnston, unpublished experiments) for reaction with OSHS and L-cysteine, which may protect the enzyme from adventitious cysteine inactivation in vivo. Under physiological conditions, thiazolidine formation is disallowed as the cofactor is tied up in aldimine linkage with the amino nitrogen of OSHS.

It is of interest, nonetheless, that cysteine provides no apparent protection against propargylglycine inactivation, and yet cysteine is completely efficient for capture of the four-carbon substrate analogue vinylglycine.[11] Perhaps the *five-carbon* propargylglycine (even as the allenic pyridoxal adduct, which ought to be reactive to thiol addition) provides a substantial steric deterrent to cysteine binding. This is a reasonable hypothesis considering that cysteine binding in the physiological reaction occurs only *after* elimination of the succinyl group from OSHS, leaving the *four-carbon* pyridoxal adduct of 2-amino-3-butenoate as the intermediate reactive for thiol addition (Johnston et al., 1979; Davis & Metzler, 1972).

Labeling Pattern. Table III summarizes the available data on protein structure relevant to the inactivation by propargylglycine of cystathionine γ-synthase, methionine γ-lyase, and γ-cystathionase, an enzyme from rat liver which bears

[11] Cystathionine γ-synthase will catalyze the synthesis of cystathionine from L-vinylglycine and L-cysteine at a rate only 50% below that for γ-replacement on the physiological substrate OSHS (Johnston et al., 1979).

mechanistic analogy[10] to the two enzymes examined in our studies.

Cystathionine γ-synthase incorporates 4 molar equiv of propargylglycine for each mol of enzyme tetramer inactivated, which argues in favor of a labeling pattern wherein each protomeric subunit suffers a single alkylation. The observed biphasic reaction kinetics, however, also suggest that α and β subunits may be nonequivalent in their rates of alkylation; this could result from either (1) differential subunit reactivity with the inactivator (either in binding or in catalysis) or (2) heterotropic negative cooperativity between subunits (α is linked to β?); both possibilities can arise from subunit heterogeneity[12] (Seydoux et al., 1974).

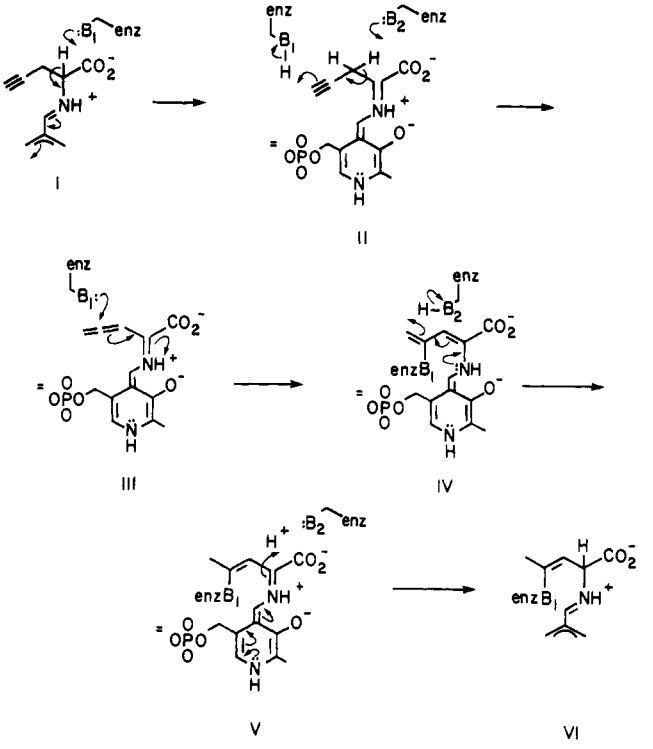
Fully inactivated methionine γ-lyase incorporates generally two, but sometimes four, labels of [^{14}C]propargylglycine per enzyme tetramer. In those experiments where half-of-the-sites reactivity is observed, the enzyme displays *absolute* or *infinite* negative cooperativity. That is, while only two monomers of the tetramer show alkylation, the unlabeled pair is totally without activity for subsequent incorporation of the label. For enzyme where four labels per tetramer eventually become incorporated, the incorporation of the first two labels gives a loss of 85% of the native catalytic activity (Figure 5)—a finding which suggests *extreme*, if not *absolute*, negative cooperativity. That is, even in the event where inactive enzyme is fully labeled, the alkylation of the first two monomers affects adversely the activity of the second two for further alkylation by the third and fourth equivalents of label. In other words, despite the stoichiometry number obtained (two to four per tetramer), the conclusion is the same: enzyme subunit activities are linked through negative cooperativity with regard to inactivation by propargylglycine.

We suspect that the reaction of propargylglycine alters radically what may be the initial catalytic equivalence of identical subunits, such that an α_2 dimer is homotropically negatively cooperative and a β_2 dimer is similarly a negatively cooperative pair. The resultant inactivation (at least through the loss of the first 80% of the enzymatic activity) gives a tetramer wherein one α subunit and one β subunit appear to become alkylated; concomitantly each alkylation all but "shuts down" the unlabeled monomer of a like-dimer pair for reaction. This hypothesis is in accord with the results for the two-dimensional gel analysis discussed above and similarly provides an explanation for biphasic inactivation kinetics. In fact, a single rate for inactivation might be expected if subunit coupling were heterotropic (α linked to β, and alkylation occurs either at two α or at two β subunits).[13]

To see whether methionine γ-lyase, completely inactive toward methionine γ-elimination (and labeled with 2 equiv of ^{14}C per tetramer), can still react with a small substrate at the unmodified subunits, tritium release from [α-^{3}H]-DL-alanine was assayed. No ^3H_2O was generated in 60 min under conditions where 180 nmol of ^{3}H was released into solvent by an equivalent amount of native methionine γ-lyase.

Half-of-the-sites reactivity, as observed for methionine γ-lyase, has now been documented for inactivation of several

Scheme I



oligomeric proteins [see Seydoux et al. (1974) for a review]; among them are rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (Levitzki, 1974), *E. coli* CTP synthetase (Levitzki et al., 1971), the two pyridoxal enzymes threonine deaminase from *Salmonella* (Hatfield & Burns, 1970) and rat liver γ-cystathionase (Washtien & Abeles, 1977), and β-hydroxydecanoylthioester dehydrase, the first enzyme inactivated by an acetylenic substrate analogue (Morisaki & Bloch, 1971).

γ-Cystathionase is a mammalian pyridoxal enzyme which bears mechanistic similarity to the bacterial methionine γ-lyase in that both catalyze elimination of a good leaving group at the γ-carbon of substrates.[10] Washtien & Abeles (1977) have examined the inactivation of γ-cystathionase by propargylglycine and have observed half-of-the-sites reactivity for labeling (note data in Table III), suggesting that the subunits are nonequivalent for reaction with propargylglycine. Biphasic kinetics for inactivation have not been observed (Abeles, personal communication). However, differential cofactor reactivity for γ-cystathionase toward reagents such as cycloserine and sodium borohydride has been reported (Churchich & Bieler, 1971). In addition, it has been shown that the enzyme binds pyridoxal phosphate differentially (K_{assoc} is 7.5 $\times$ 10^5 and 8.3 $\times$ 10^4 M^{-1}; Churchich et al., 1975), which implies distinct microenvironments for the two pairs of cofactors in the tetrameric protein.

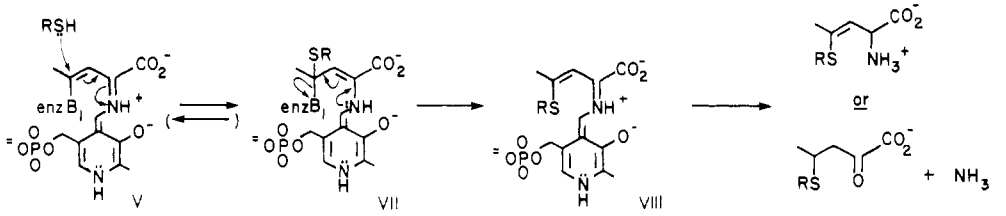
The subunits of γ-cystathionase are presumed to be identical (Deme et al., 1972). It will be of interest to know whether this enzyme also gives evidence for microheterogeneity of subunit composition when subjected to two-dimensional electrofocusing/electrophoresis.

Trifluoroalanine also inactivates γ-cystathionase (Silverman & Abeles, 1976, 1977) with two labels incorporated per enzyme tetramer. Enzyme which has been inactivated by 2 mol of propargylglycine per tetramer still reacts with trifluoroalanine; 2 mol of trifluoroalanine becomes covalently bonded per mol of tetramer. This is a result which argues against *infinite* negative cooperativity between subunits for

[12] We have not yet determined whether the observed microheterogeneity of the two enzymes examined here results from substantial differences in amino acid composition and sequence or reflects rather only minor alterations (such as N-terminal blocking or posttranslational modification) in otherwise identical subunits.

[13] It is possible that native enzyme contains only two *reactive* active sites per tetramer. However, the pyridoxal content determined (four molecules per tetramer) and the fact that the stoichiometry of inactivation is sometimes observed to be four labels per tetramer are both findings which argue for four *active* active sites per tetrameric protein.

Scheme II



alkylation of γ-cystathionase. Although we have yet to examine the reaction of methionine γ-lyase with trifluoroalanine, we predict that propargylglycine-inactivated enzyme will be unreactive to additional alkylations by trifluoroalanine, based on the enzyme's inability to abstract the α-proton from L-alanine.

Product Formation. We have not yet identified the product(s) formed in the very few successful turnovers of propargylglycine by either methionine γ-lyase or cystathionine γ-synthase. Marcotte & Walsh (1978a) have recently shown that the flavoenzyme D-amino-acid oxidase carries out the oxidation of D-propargylglycine to yield 2-imino-4-pentynoate. This product then suffers a number of nonenzymatic rearrangements which have been characterized in detail (Marcotte & Walsh, 1978b). The accumulating product of the enzymatic oxidation is a cyclic amine diene lactone.

We predict that both the pyridoxal enzymes studied here may support 2e-oxidation at C_2 and 2e-reduction at $C_{4,5}$ of the enzyme-bound allenic structure (III of Scheme I). Two potential products are likely in this event, 2-keto-3-pentenoate (**3**) and 2-keto-4-pentenoate (**4**) (Marcotte & Walsh, 1978c).

3 **4**

By the criterion of "turnovers per inactivation event" (Walsh et al., 1978), propargylglycine, at 4:1 and 6–8:1 for cystathionine γ-synthase and methionine γ-lyase, respectively, is a highly efficient suicide substrate in these inactivations. These are notably small partition ratios when compared, for instance, to those for D-vinylglycine inactivation of D-amino-acid transaminase (450–800:1; Soper et al., 1977) and for D-fluoroalanine-induced inactivation of alanine racemase (800:1; Wang & Walsh, 1978).

Inactivation Mechanism. We propose in Scheme I a mechanism for inactivation of both cystathionine γ-synthase and methionine γ-lyase which involves a minimum number of active-site residues (acting both as bases and as nucleophiles) consistent with irreversible alkylation of each enzyme. It is essentially the mechanism proposed previously for propargylglycine inactivation of γ-cystathionase (Abeles & Walsh, 1973). We have previously given evidence for a minimum two-base mechanism for the reaction of cystathionine γ-synthase with its substrates and substrate analogues (Johnston et al., 1979).

Although no direct evidence exists for conjugated allene formation in the propargylglycine reactions, a wealth of circumstantial evidence points toward it as a reactive electrophile in enzymatic inactivation by acetylenes [see Walsh (1978a) for a review] and in model reactions (Morisaki & Bloch, 1971; Batzold & Robinson, 1975, 1976).

We suspect that, following alkylation, the modified enzyme adduct (structure IV) will rapidly tautomerize the exomethylene double bond into conjugation with the ketimine quinoid (structure V). Reprotonation of the α-carbon gives irreversibly alkylated enzyme.

Structure VI may be expected to give visible absorbance which is more or less identical with that for active enzyme in its pyridoxaldimine form. It is precisely because the spectra of Figure 8 are without remarkable chromophoric patterns (the A_{422} is reduced by about 20%) that we suggest that VI may represent the structure of fully inactivated cystathionine γ-synthase. (It is also the likely adduct accumulating in γ-cystathionase inactivation; Washtien & Abeles, 1977.) In this regard, note that conversion of V to VI requires a proton not available (at least in the formal sense) at the active site. One may postulate, therefore, that $EnzB_2$: is able to exchange protons with solvent water.[14]

If, however, $EnzB_2$: is sequestered from bulk solvent, then reprotonation to VI may not be facile and V will accumulate. Structure V is a pyridoximine quinoid with extended β,γ-conjugation and may be expected to give high-wavelength absorbance (Walsh, 1978b). This is precisely what is observed in Figure 9, and thus we suggest that V represents a reasonable structure for inactivated methionine γ-lyase. In this case, methionine γ-lyase must not accomplish appreciable reprotonation at the α-carbon; this is in accord with the physiological mechanism, where γ-elimination of methanethiol gives release of aminocrotonate *without* reprotonation at the α-carbon of the PLP-bound intermediate (Davis & Metzler, 1972). By contrast, cystathionine γ-synthase accomplishes an elimination–replacement sequence where reprotonation at the α-carbon of the PLP-bound product is an *obligate* mechanistic event in the γ-replacement pathway.

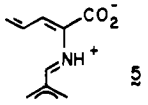
In an attempt to test these structural assignments, we conducted the thiol reactivation studies described above, based upon the prediction that V is still susceptible to nucleophilic attack and enzymatic reactivation through an addition–elimination sequence as outlined in Scheme II. The tetrahedral adduct (VII) could either eliminate RS^- or, perhaps, the enzymic nucleophile ($EnzB_1$) which formed the inactivating covalent bond. In the later event, VIII may be processed catalytically (elimination or replacement mode) to give reactivated enzyme.

Note for structure VI that the $C_{3,4}$ double bond is out of conjugation, because C_2 is at the amine oxidation state, and ought to be, therefore, unreactive to the addition–elimination sequence. Consistent with this prediction, cystathionine γ-synthase experiences no thiol reactivation while methionine γ-lyase is reactivated by thiols, with an average recovery of 25% catalytic activity. We have not yet attempted to detect any sulfur-containing product(s) from this substoichiometric reactivation.

Further support for the assignment of V to the inactivated methionine γ-lyase is the observation that the reactivation is accompanied by bleaching of the high-wavelength absorbance bands and recovery of the 418-nm pyridoxaldimine absorbance. However, full enzymatic activity is not recovered by thiol treatment nor is a spectral pattern regained which is isospectral

[14] Or some other active-site group, not $EnzB_2$, provides the needed proton.

with fully active enzyme. Partial reactivation could derive from at least two possibilities: (1) initial alkylation, with complementing fractional stoichiometry, by two *different* nucleophiles at any given active site of differing reactivity for intramolecular elimination from the tetrahedral adduct VII or (2) partitioning of the allene paraquinoid III between alkylation and tautomerization to a pyridoxaldimine adduct of 2-enamino-4-pentenoate (**5**), which is also a potential al-



5

kylating agent if $EnzB_1$ attacks the vinylogous center at C_5. If the latter possibility is obtained, the resultant adduct may suffer *reversible* addition–elimination of thiol at C_4, but this will not labilize the inactivating bond between C_5 and an enzymic base. Structural elucidation of the adducts will be required to determine the site(s) of attack[15] and the nature and number of the active-site nucleophiles involved for both cystathionine γ-synthase and methionine γ-lyase.

Finally, the inactivation data reported here for cystathionine γ-synthase are consistent with our mechanistic prediction that this γ-replacement enzyme would be the target enzyme in bacterial methionine biosynthesis and may represent the major, if not the sole, in vivo killing site of this naturally occurring γ,δ-acetylenic α-amino acid.

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[15] It is C_4 in γ-cystathionase (Washtien & Abeles, 1977).