Mechanistic Studies with Vinylglycine and β-Haloaminobutyrates as Substrates for Cystathionine γ-Synthetase from *Salmonella typhimurium*†

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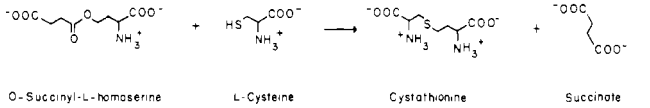
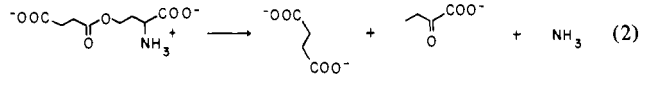
ABSTRACT: Cystathionine γ-synthetase (EC 4.2.99.9), a key enzyme in bacterial methionine biosynthesis, has been found to use L-vinylglycine (2-amino-3-butenoate) and L-β-haloaminobutyrates (X = F, Cl) as substrates in addition to the physiological γ-substituted substrate *O*-succinyl-L-homoserine (OSHS). Vinylglycine is a substrate both for α-ketobutyrate formation (the normal product from γ elimination with OSHS) and for cystathionine formation (the normal γ-replacement product with OSHS) in the presence of cysteine. This behavior substantiates that the stabilized vinylglycine–pyridoxal phosphate (PLP) α carbanion is the key partitioning species in this enzyme's catalysis. The V_{max} values for ketobutyrate production and cystathionine formation from vinylglycine are equivalent at approximately 45 U/mg, whereas the corresponding V_{max} values from OSHS are 20 and 200 U/mg, respectively, suggesting different rate-determining steps with these two substrates. The β-haloaminobutyrates undergo catalyzed HX elimination to yield bound aminocrotonate–PLP directly as an initial intermediate and as a precursor of ketobutyrate. Little or no cystathionine formation is detectable when these substrates are incubated with enzyme and the normal cosubstrate cysteine, strongly indicating that the aminocrotonate–PLP intermediate is not in rapid, reversible equilibrium with the stabilized vinylglycine–PLP carbanion; in normal catalysis, the prototropic shift from α carbanion to aminocrotonate appears functionally unidirectional. The HX-elimination step from β-chloroaminobutyrate is nonconcerted as demonstrated by a $^3H_2O \rightleftharpoons$ chloroaminobutyrate exchange reaction. Further suggestion for discrete β-halo-α-carbanionic intermediates derives from the observation that the haloaminobutyrates appear to partition between ketobutyrate formation and enzyme inactivation. Since neither vinylglycine nor OSHS causes any detectable inactivation during turnover, it is likely that the inactivation species is not a common intermediate, i.e., the electrophilic aminocrotonate–PLP species (a potential Michael acceptor), but rather a species peculiar to the β-haloaminobutyrate pathway. The β-halo-α-carbanion–PLP intermediate has β-halo-α-iminodihydropyridine character in the *p*-quinoid resonance contributor and is a good candidate for an alkylating agent by an S_N2-displacement mechanism. Spectroscopic analyses of incubations with the various amino acid substrates show a number of long-wavelength absorbing species forming during turnover; tentative assignments are suggested.

Cystathionine γ-synthetase is a key enzyme in microbial and plant methionine biosynthesis; it catalyzes the net formation of cystathionine from *O*-succinyl-L-homoserine (OSHS)[1] and cysteine (reaction 1). In the absence of cysteine, the enzyme



(1)

catalyzes elimination of succinate and isomerization to give α-ketobutyrate and ammonia (reaction 2).



(2)

Cystathionine γ-synthetase has been purified to homogeneity from *Salmonella typhimurium* by Flavin and his colleagues (Kaplan & Flavin, 1966; Guggenheim & Flavin, 1969a; Kaplan & Guggenheim, 1971); the protein is a tetramer of four identical subunits (mol wt 40 000) each containing one residue of tightly bound pyridoxal 5-phosphate.

Pyridoxal assisted γ elimination and γ replacement are assumed to account for both reactions 1 and 2 by a mechanism outlined in Scheme I. Substrate OSHS undergoes (1) conventional transaldimination with enzyme–pyridoxal; (2) α-proton abstraction to yield the α carbanion stabilized as a pyridoxalketimine *p*-quinoid; and (3) β-proton removal, which leads to succinate elimination and formation of a β,γ-unsaturated *p*-quinoid III.[2]

Structure III is thought to be a key intermediate for all enzymes which accomplish pyridoxal-assisted γ eliminations and γ replacements (Davis & Metzler, 1972). It may suffer (4) addition of a nucleophile at the methylene carbon, as in the case of cystathionine synthesis; alternatively (5) it may isomerize to the eneamino pyridoxal aldimine (IV),[2] which in turn gives rise (6) to α-ketobutyrate and ammonia.

This mechanism predicts that the olefinic amino acid vinylglycine (2-amino-3-butenoate) should serve as a substrate

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[1] Abbreviations used: OSHS, *O*-succinyl-L-homoserine; NADH, nicotinamide adenine dinucleotide, reduced form; KP_i, potassium phosphate; KPP_i, potassium pyrophosphate; LDH, lactate dehydrogenase; NbS_2, 5,5′-dithiobis(2-nitrobenzoate); PLP, pyridoxal phosphate.

[2] Structures III and IV of Scheme I are numbered so as to correspond with those given in the proposed general Scheme III, presented in the Discussion section.

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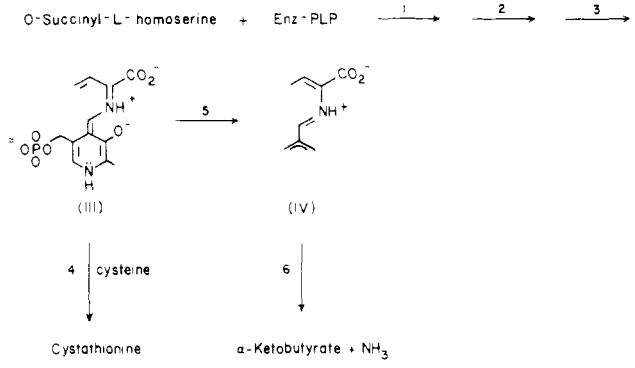
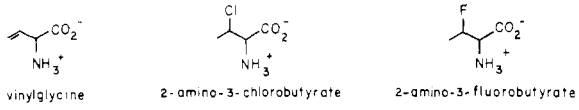
Scheme I



for both α-ketobutyrate formation and (in presence of cysteine) for cystathionine synthesis, since the key intermediate (III) is merely the stabilized α carbanion of vinylglycine. Similarly, the two β-haloamino acids, 2-amino-3-chlorobutyrate and 2-amino-3-fluorobutyrate, should give α-ketobutyrate formation if α-proton abstraction leads to elimination of the halide and formation of IV. The ability of the enzyme to support cystathionine synthesis from the two haloaminobutyrates might be expected to depend upon the position of the equilibrium between compounds III and IV at the active site and upon the rate of isomerization of IV to III vs. the rate of hydrolysis of IV. We report here on the reactions of these substrate analogues with cystathionine γ-synthetase.



Materials and Methods

Enzyme Purification. Cystathionine γ-synthetase was purified from extracts of *Salmonella typhimurium* me-A (ATCC 25241) by modification of the procedures of Guggenheim & Flavin (1969a). Their ammonium sulfate fractionation (step 5) was omitted. Chromatography on DEAE-cellulose and on hydroxylapatite (steps 6 and 7) was followed by gel filtration (Sephadex G-200). The enzyme preparation was found to be homogeneous by acrylamide gel electrophoresis and by isoelectrofocusing. The specific activity against OSHS was seen to be slightly higher (20 U/mg, in the γ-elimination assay described below) than reported previously (18.2 U/mg, Kaplan & Guggenheim, 1971). The holoenzyme at pH 7.3 has two absorbance maxima, at 280 nm and at 422 nm. The ratio A_{280}/A_{422} was 3.90; Kaplan & Guggenheim (1971) reported $A_{280}/A_{422} = 3.85$ for purified enzyme.

Preparation of Substrates, Substrate Analogues, and Inhibitors. *O*-Succinyl-L-homoserine (OSHS) was either prepared according to Flavin (1971) or purchased from Sigma. DL-Vinylglycine (2-amino-3-butenoate) was prepared according to the method of Baldwin et al. (1977) and was then resolved by treatment of *N*-chloroacetyl-DL-vinylglycine with hog kidney acylase I (Sigma).

α-DL-[^{3}H]Vinylglycine was prepared by F. Jacobson in this laboratory, using arginine racemase partially purified (Yorifugi et al., 1971a,b) from *Pseudomonas taetrolens* (ATCC 4683), which will catalyze exchange of the α proton with solvent tritium. The reaction was carried out as follows. To 0.5 mL of 10 mM KP_i buffer, pH 7.3, were added 3.0 units of racemase (ca. 10 U/mg), 10 mg of DL-vinylglycine (100 μmol), and 100 μL of ^3H_2O (1 Ci/mL). The reaction was incubated at 37 °C for 5 h. The solution was then lyophilized repeatedly; the residue was dissolved in 0.5 mL of water and loaded onto a Dowex 50 H^+ column. The column was washed with water until the tritium levels fell to background and was then eluted with 2 N NH_4OH. The fractions containing radioactivity were pooled and lyophilized. A single radioactive ninhydrin spot cochromatographed with authentic vinylglycine. This procedure gave α-[^{3}H]vinylglycine of specific activity 755 cpm/nmol.

(2*R*,3*R*)-2-Amino-3-chlorobutyrate (*erythro*-L-chloroaminobutyrate)[3] was prepared by the methods of Cheung & Walsh (1976).

(2*R*,3*R*)-2-Amino-3-fluorobutyrate (*erythro*-L-fluoroaminobutyrate) and (2*R*,3*S*)-2-amino-3-fluorobutyrate (*threo*-L-fluoroaminobutyrate) were gifts of Dr. J. Kollonitsch and colleagues of Merck, Sharp and Dohme Research Laboratories.

Radiolabeled L-cysteine was prepared by dithiothreitol reduction of L-[3,3′-^{3}H]cystine, as outlined by Datko et al. (1974); tritiated cystine was obtained from Amersham. All scintillation counting was performed using ACS scintillation fluid.

β-Chloro-L-alanine was purchased from Vega Fox Biochemicals and was used without further purification.

Kinetic Assays. The continuous assay of α-ketobutyrate formation (reaction 2) from substrate OSHS (γ elimination) or from substrate analogues was accomplished by reduction of keto acid in the presence of NADH and lactate dehydrogenase (Boehringer-Mannheim, from rabbit skeletal muscle). A standard 1.0-mL assay contained 50 mM KPP_i buffer, pH 8.3 at 37 °C, an appropriate substrate concentration, 2 μg of purified cystathionine γ-synthetase, 0.3 mM NADH, and 0.3 mg of LDH.

The synthesis of cystathionine (reaction 1) either from OSHS (γ replacement) or from substrate analogues was assayed discontinuously, using a standard NbS_2 thiol consumption assay. A 1.0-mL assay contained 50 mM KPP_i buffer, pH 8.3 at 37 °C, and appropriate substrate concentrations (including cysteine) and 2.0–10.0 μg of purified cystathionine γ-synthetase. Aliquots (100 μL) were withdrawn at timed intervals to the NbS_2 reagent (900 μL of 0.2 mM NbS_2 in 100 mM KP_i buffer, pH 7.3). Cysteine consumption was determined by ΔA_{412} vs. time.

The rate of enzyme-catalyzed proton exchange between α-[^{3}H]vinylglycine and solvent water was determined in the following way. A 1.5-mL solution was prepared containing 80 mM α-[^{3}H]vinylglycine (755 cpm/nmol), 0.6 mM NADH, and 1.0 mg of LDH, all in 50 mM KPP_i buffer, pH 8.3 at 37 °C. The reaction was initiated by addition of 2.0 μg of partially purified (4.5 U/mg) cystathionine γ-synthetase, and ketobutyrate formation was continuously monitored as disappearance of absorbance at 340 nm. At timed intervals, 100-μL aliquots were withdrawn from the reaction cuvette 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 scintillant. This procedure allowed the simultaneous determination in a single assay of the rates of both ketobutyrate production and of tritium "washout to solvent".

The ability of cystathionine γ-synthetase to catalyze hydrogen exchange between solvent protons and chloroaminobutyrate was evaluated in the following way. A solution was prepared which contained (2*R*,3*R*)-2-amino-3-chlorobutyrate (25 mM) and ^3H_2O (100 mCi) in KPP_i buffer (50 mM, pH

[3] In the Cahn–Ingold–Prelog system for assigning absolute configuration, the L stereoisomers of β-halo-α-amino acids assume the *R* designation at the chiral α carbon, as halogens take priority over oxygen.

Table I: Kinetic Parameters for the Reactions of Cystathionine γ-Synthetase with Substrates and Substrate Analogues

compound	reaction 1		reaction 2	
	K_M(Cys) (mM)	V_{max} (μmol min^{-1} mg^{-1})	K_M (mM)	V_{max} (μmol min^{-1} mg^{-1})
O-succinyl-L-homoserine	0.23	192	0.18	21.0
vinylglycine	0.40	46	5.2	44.2
(2*R*,3*R*)-2-amino-3-chlorobutyrate (*erythro*-L-chloroaminobutyrate)	0.0	0.0	1.3	1.2
(2*R*,3*R*)-2-amino-3-fluorobutyrate (*erythro*-L-fluoroaminobutyrate)	n.d.[a]	(0.19)	1.5	3.3
(2*R*,3*S*)-2-amino-3-fluorobutyrate (*threo*-L-fluoroaminobutyrate)	n.d.[a]	(0.30)	1.9	2.7
β-chloro-L-alanine	0.70	8.9	2.9	8.0

[a] n.d. = not determined.

8.3); the final reaction volume was 200 μL. Reaction was initiated by addition of 0.01 mg of enzyme. After 5-min incubation at 37 °C, the reaction was quenched by immersion in a boiling water bath for 5 min. The solution was then Millipore-filtered and lyophilized repeatedly (ten times in 50-μL volumes) to remove excess ^3H_2O until the residual radioactivity had fallen to a constant value (approximately 22 000 cpm). After the final lyophilization, the residue was applied to a silica gel thin-layer sheet and chromatographed (butanol–acetic acid–water, 4:1:1) against authentic chloroaminobutyrate. After chromatography, all the radioactivity was seen to migrate with authentic chloroaminobutyrate (R_f 0.31, visualized by ninhydrin spray).

Inactivation Kinetics. Inactivation experiments involved the following protocol. At time zero, 20 μL of purified cystathionine γ-synthetase (0.95 mg/mL) was added to 80 μL of a solution containing an appropriate concentration of either (2*R*,3*R*)-2-amino-3-chlorobutyrate or (2*R*,3*R*)-2-amino-3-fluorobutyrate; the final incubation was 50 mM KPP_i buffer, pH 8.3 at 37 °C. At intervals, 10-μL aliquots were removed and assayed by dilution to cuvettes containing 1 mL of 5 mM OSHS, 50 mM KPP_i, pH 8.3 at 37 °C, 0.3 mM NADH, and 0.3 mg of LDH, permitting continuous assay of the rate of formation of α-ketobutyrate.

Spectral Studies. Steady-state absorbance spectra for the reaction of cystathionine γ-synthetase with substrates and substrate analogues were obtained using a Perkin-Elmer Hitachi 200 scanning spectrophotometer. A typical spectral analysis involved the following protocol. An initial absorbance scan was made on a solution of cystathionine γ-synthetase (usually 475 μg) in KPP_i buffer, pH 8.3. At time zero, 50 μL of substrate or analogue was added to bring the reaction volume to 500 μL and the buffer concentration to 50 mM, pH 8.3. The enzyme concentration was thus approximately 0.5 mg/mL and the substrate or substrate analogue saturating (the concentration of which was dictated by the K_M value determined from kinetic analyses). Absorbance scans were made at timed intervals following the addition of substrate. The spectral experiments were always carried out at 4 °C to prolong the period of steady-state analysis. Guggenheim & Flavin (1971) have shown that the K_M for OSHS (0.3 mM) determined at 37 °C is unchanged at 5 °C; the maximum velocity of γ elimination, however, was shown to be 22 times greater at 37 °C (20 μmol min^{-1} mg^{-1}) than at 5 °C.

Results

I. Vinylglycine

(*a*) *Kinetic and Product Analysis.* The kinetic data for cystathionine γ-synthetase catalyzed cystathionine synthesis (reaction 1) and α-ketobutyrate formation (reaction 2) for substrate *O*-succinyl-L-homoserine (OSHS) and for vinylglycine and other substrate analogues are given in Table I. Kaplan & Flavin (1966) report a V_{max} for substrate (OSHS) γ replacement (93.8 μmol min^{-1} mg^{-1}) which is approximately fivefold greater than the V_{max} for γ elimination (18.8 μmol min^{-1} mg^{-1}). We observe a nearly tenfold increase in V_{max} for replacement over elimination; the bases for this difference are unclear.

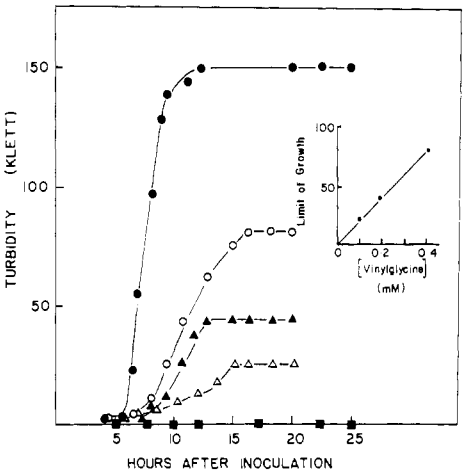


FIGURE 1: Effect of 0.1 mM methionine (●) and 0.1 mM (△), 0.2 mM (▲), and 0.4 mM (○) vinylglycine on growth of *S. typhimurium* me-A. The cultures were prepared according to Delavier-Klutchko & Flavin (1965). Control cultures containing minimal medium and lacking both methionine and vinylglycine give no growth (■). Cell growth (at 37 °C) was monitored as optical density and is reported in Klett units.

Table I indicates that cystathionine γ-synthetase is able to support both keto acid formation (reaction 2) and cystathionine synthesis (reaction 1) from vinylglycine. We have obtained both in vivo and chemical evidence that the enzyme-catalyzed thiol consumption observed in the presence of vinylglycine represents authentic cystathionine synthesis. The in vivo evidence comes from the observation that vinylglycine is an effective substitute for methionine, which must otherwise be added to cultures for growth of the *Salmonella* mutant me-A (Delavier-Klutchko & Flavin, 1965). Figure 1 shows the effect of added vinylglycine on the growth of this *S. typhimurium* methionine mutant; note that the cell density (as expressed in Klett units) at stationary phase is linear with vinylglycine concentration (inset). These data are consistent with the ability of cystathionine γ-synthetase to catalyze cystathionine formation by γ replacement of thiol upon vinylglycine. Note from Figure 1 that, even at 0.4 mM vinylglycine, maximal growth is only about 50% of that obtained for cultures incubated with 0.1 mM methionine. It is probable that vinylglycine acts as a substrate in vivo for a variety of enzymes in addition to cystathionine γ-synthetase.[4]

Cystathionine γ-synthetase similarly accomplishes cystathionine biosynthesis in vitro from vinylglycine and isotopically labeled [^{3}H]cysteine. When the partially purified enzyme (8.0 U/mg) is incubated with excess L-vinylglycine (20 mM) and [^{3}H]-L-cysteine (10 mM), all of the radioactivity is isolated on paper chromatograms as a single ninhydrin-reactive spot which cochromatographs with authentic cystathionine (R_f 0.12; butanol–acetic acid–water, 12:3:5).

(*b*) *Reversible Exchange of Vinylglycine α Hydrogen with Solvent.* Cystathionine synthesis (reaction 1) is observed to be appreciably slower from vinylglycine than from substrate OSHS. Moreover there is no evidence for any rate enhancement of reaction 1 over reaction 2 in the case of vinylglycine (44 vs. 46 U/mg) which is observed to be nearly tenfold for substrate OSHS. Further, the V_{max} value for ketobutyrate formation from vinylglycine is approximately twice (44 vs. 20 U/mg) that observed for OSHS. It may be that γ elimination of the succinyl residue is at least partially rate determining for reaction 2, a chemical event obviated in ketobutyrate formation from vinylglycine.

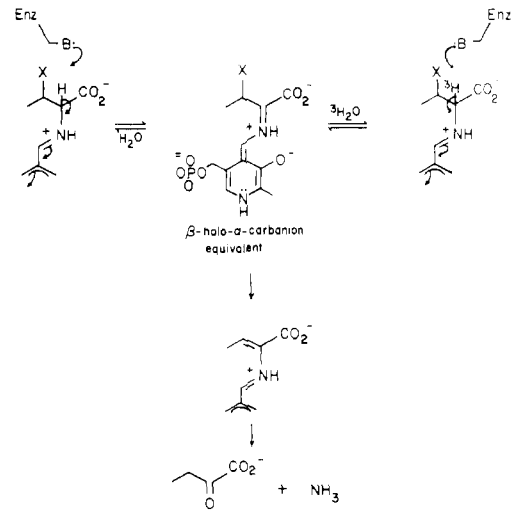
Supportive of this conclusion are the observations of Flavin and his colleagues regarding the ability of cystathionine γ-synthetase to catalyze rapid proton-exchange reactions with solvent protons. The enzyme, for example, will catalyze exchange between solvent tritium and both α and β hydrogens of a variety of amino acids, with rates favoring more rapid exchange at the β position if the γ carbon bears an electronegative substituent (Posner & Flavin, 1972a). Cystathionine itself suffers slow γ elimination and hydrogen exchange with solvent at the α and β carbons on both sides of the thioether linkage. The hydrogen exchange rates were observed to be about 10^3 times faster than the rates of cysteine exchange and of elimination (Guggenheim & Flavin, 1969b), results which indicate (1) that the β,γ-elimination step (of H^+ and succinate) is nonconcerted and (2) that both α- and β-carbanion formation occur many times for each γ-succinyl group lost.

With these observations in mind, we have determined the rate of tritium washout to solvent from α-[^{3}H]vinylglycine using partially purified cystathionine γ-synthetase (4.5 U/mg); in a given assay the rate of ^3H_2O formed at saturating vinylglycine was 27.4 nmol min^{-1}. The corresponding rate of ketobutyrate formation was 15.9 nmol min^{-1}. This is a velocity ratio for ^3H_2O to ketobutyrate formation of 1.72, confirming that α-proton abstraction is relatively rapid and—as similarly observed for substrate OSHS—cannot be rate determining in the turnover of vinylglycine.

II. β-Halo-α-aminobutyrates

(*a*) *Kinetic Analyses.* All of the β-haloamino acids of Table I give enzyme-catalyzed ketobutyrate formation (reaction 2) at rates substantially below those determined for either OSHS or for vinylglycine; the three-carbon β-chloroalanine gives the highest value. This latter reaction (which yields pyruvate) is equivalent to β elimination from succinylserine originally reported by Guggenheim & Flavin (1969a), which proceeds with an identical V_{max} = 8.0 μmol min^{-1} mg^{-1}. β replacement on succinylserine by homocysteine was observed by these workers at a rate about 0.2% (0.2 μmol min^{-1} mg^{-1}) that obtained for γ replacement on OSHS by cysteine. For chloroalanine, the V_{max} (8.9 μmol min^{-1} mg^{-1}) for thiol consumption (presumed β replacement) reported here is about 5% of that for γ replacement on substrate OSHS. Guggenheim & Flavin (1969a) observed that the enzyme becomes partially inactivated while catalyzing pyruvate formation from succinylserine. We have observed in preliminary experiments that chloroalanine also inactivates cystathionine γ-synthetase (vide infra).

Scheme II



The formation of α-ketobutyrate (reaction 2) from either diastereomer of L-fluoroaminobutyrate proceeds with values for V_{max} (3.3 and 2.7 μmol min^{-1} mg^{-1}) which are both more than twice that observed for keto acid formation from the *erythro*-L-chloroaminobutyrate. Recently Wang & Walsh (1978) have shown that halide-ion elimination catalyzed by alanine racemase proceeds some 50-fold faster from either L- or D-fluoroalanine than from L-chloroalanine. While one might suspect that the rate of elimination may be in part controlled by the absolute stereochemistry of the leaving halide in relation to the α proton,[5] it would also appear that differential halide reactivity (fluorine over chlorine, in the case of the intramolecular eliminations described here) is also influential of rate. Thus, our data tend to suggest that ketobutyrate formation proceeds by a relatively slow, and partially rate-determining, halide elimination from a stabilized β-halo-α-carbanion.

(*b*) *Reversible Exchange of α Hydrogen with Solvent.* Since cystathionine γ-synthetase catalyzes rapid α-proton exchange both from physiological substrates (Guggenheim & Flavin, 1969b) and from vinylglycine (this report), a slow halide-elimination step predicts the detection of an α-tritiohaloaminobutyrate as an experimental finding in ^3H_2O diagnostic of a putative β-halo-α-carbanion which forms reversibly (and discretely antecedent to halide loss) during turnover of the two haloamino acids by the enzyme. This argument is illustrated in Scheme II. In an attempt to verify this prediction, we have carried out the reaction of (2*R*,3*R*)-2-amino-3-chlorobutyrate (25 mM) in the presence of enzyme (0.01 mg) and ^3H_2O (100 mCi). After the workup outlined above in Materials and Methods, the total radioactivity recovered was estimated to represent the incorporation of about 2.4 nmol of tritium into chloroaminobutyrate in 5 min. Given a V_{max} for ketobutyrate formation of 1.2 μmol min^{-1} mg^{-1} (Table I), the amount of ketobutyrate formed during the same 5-min incubation is estimated to be about 60 nmol. (This corresponds to about 10% substrate conversion, assuming that initial velocity is

[4] Recently, for example, Soda and his colleagues (Esaki et al., 1977) have shown that a *Pseudomonas* methionine γ-lyase will catalyze turnover of vinylglycine to α-ketobutyrate.

[5] Indeed, L-chloroalanine is processed by alanine racemase at a substantially lower rate than the corresponding D compound (Wang & Walsh, 1978).

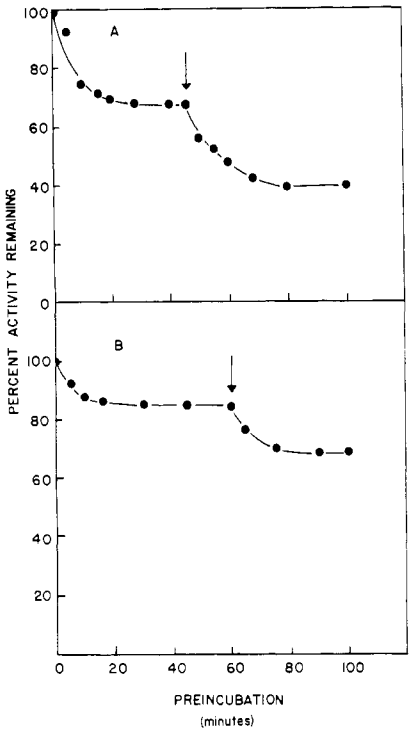


FIGURE 2: Kinetics of inactivation of cystathionine γ-synthetase by (A) 25 mM (2*R*,3*R*)-2-amino-3-chlorobutyrate and by (B) (2*R*,3*R*)-2-amino-3-fluorobutyrate. The arrows indicate the time at which a second addition of the haloamino acids was made to the preincubation mixtures.

maintained.) Thus one obtains from these data a ratio of 1:25 for tritium "wash-in to substrate" vs. ketobutyrate formed,[6] which suggests the reversible formation of a discrete β-halo-α-carbanion in the pathway for ketobutyrate synthesis.

(*c*) *Lack of Reaction with Cosubstrate Cysteine.* No detectable thiol consumption is observed for reaction of the enzyme with chloroaminobutyrate and cysteine. Both diastereomers of fluoroaminobutyrate, however, promote detectable, although exceedingly slow, disappearance of cysteine at rates which are less than 0.2% of that observed for γ replacement on OSHS and less than 1% of the rates for ketobutyrate formation from either of the fluoroamino acids. These slow rates require large amounts of both isomers of fluoroaminobutyrate for a precise kinetic analysis, and, thus, the V_{max} values given are only estimates of maximal velocity. No discrimination between the two diastereomers of L-fluoroaminobutyrate is evident for either reaction 1 or reaction 2.

(*d*) *Enzyme Inactivation and Partition Ratios.* During the course of determining the kinetics for ketobutyrate formation from the two haloaminobutyrates, it was observed that the enzyme suffers slow inactivation. It appears that both chloro- and fluoroaminobutyrates partition between rapid turnover to α-ketobutyrate and a much slower inactivation of cystathionine γ-synthetase. The kinetics of inactivation by (2*R*,3*R*)-2-amino-3-chlorobutyrate and by (2*R*,3*R*)-2-amino-3-fluorobutyrate are given in Figure 2. In the case of the chloro compound, the amount of inactivation attains a plateau at about 65% residual activity after about 15 min of incubation; this level and the rate of approach to it are consistent with exhaustion of the chloroaminobutyrate by keto acid formation. Note that additional inactivation is achieved if the solution is again made 25 mM in chloroaminobutyrate. A similar pattern is observed for the fluoroaminobutyrate, save that the levels of final residual activity are higher than for the chloroaminobutyrate inactivation. The pseudo-first-order rate constant for partial inactivation by chloroaminobutyrate is 5.8×10^{-4} s^{-1}; the partition ratio between turnover of the chloroaminobutyrate to ketobutyrate and an inactivation event is 1320:1. Partial inactivation by the fluoroaminobutyrate proceeds with a pseudo-first-order rate constant of 2.8×10^{-4} s^{-1}; the partition ratio for turnover to inactivation is 4200:1. The difference in the values of the two ratios argues *against* partitioning for the two compounds from a common intermediate. No inactivation was seen during enzymic processing of vinylglycine or of OSHS in either reaction 1 or 2. No structural studies on inactivated enzyme have yet been attempted.

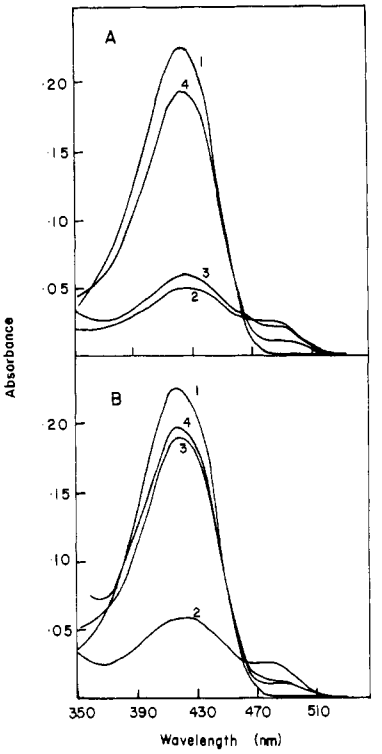


FIGURE 3: Absorbance spectra of cystathionine γ-synthetase catalyzed γ elimination (A) and γ replacement (B) on OSHS. (See text for reaction conditions.) Curves A1 and B1 are obtained prior to the addition of substrates. Curves A2, A3, and A4 obtained 1, 6, and 10 min after addition of OSHS (10 mM). Curve B2 obtained 1 min after addition of OSHS (10 mM). Curves B3 and B4 obtained 1.5 and 3.0 min after the addition of L-cysteine (5 mM). The enzyme concentration was 0.95 mg/mL (5.9 nmol).

III. Steady-State Spectroscopic Studies

(*a*) *O-Succinyl-L-homoserine.* The absorbance spectra for cystathionine γ-synthetase catalyzed γ elimination and γ replacement on substrate OSHS are presented in Figure 3. Prior to addition of substrates, the enzyme gives spectra (3A1 and B1) characteristic of enzyme-bound pyridoxaldimine 5-phosphate; there is a single visible band at 422 nm. In the reaction of OSHS and enzyme (γ elimination), addition of substrate produces a quenching of the 422-nm band (which is also slightly red shifted) and the development of a new chromophore of λ_{max} about 485 nm. Guggenheim & Flavin (1971) have previously reported this spectral behavior. As the reaction emerges from steady state, the 485-nm band dissipates and the 422-nm chromophore recovers until a spectrum (3A4), nearly identical with that for native enzyme (3A1), is obtained.

[6] This value expresses a *minimum* rate ratio which may be perturbed both by an undetermined primary solvent kinetic isotope effect and by the degree to which the exchanging base at the active site of the enzyme is sequestered from bulk solvent.

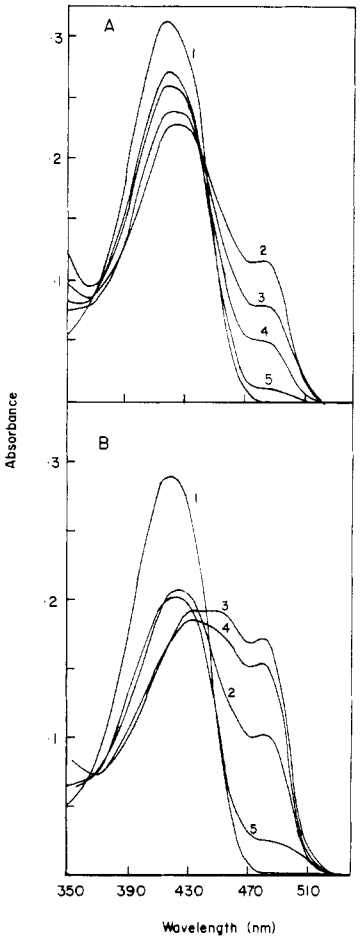


FIGURE 4: Absorbance spectra of cystathionine γ-synthetase catalyzed turnover of vinylglycine to α-ketobutyrate (A) and to cystathionine (B). (See text for reaction conditions.) Curves A1 and B1 are obtained prior to addition of substrates. Curves A2–A5 were generated 1, 5, 8, and 12 min after addition of vinylglycine (10 mM). Curve B2 similarly was obtained 1 min after addition of vinylglycine. Curves B3–B5 were obtained 1, 4, and 20 min after addition of L-cysteine (5 mM). The enzyme concentration was 1.05 mg/mL (6.5 nmol).

The final A_{422nm} value represents a 10% dilution of the enzyme following the addition of substrate.

The spectra for γ elimination are notably (and reproducibly) nonisosbestic, which might argue for more than two kinetically competent species at steady state. Guggenheim & Flavin (1971) report, however, a clearly defined isosbestic point between the two chromophores at 460 nm.

The steady-state spectra for γ replacement (Figure 3B) on OSHS (10 mM) by limiting cysteine (5 mM) are remarkably similar to those obtained for γ elimination (Figure 3A). The addition of OSHS to enzyme produces the characteristic development of a 485-nm absorbance and quenching of the 422-nm band (curve 3B2). Addition of cysteine produces, however, a very rapid recovery of the 422-nm chromophore (essentially complete within 3 min) which is not accompanied by immediate loss of the 485-nm absorbance (curves 3B3 and 3B4).

Guggenheim & Flavin (1971) report that cysteine addition gives rapid recovery of the 422-nm band *and* simultaneous bleaching of the 485-nm chromophore. The high wavelength band is not seen again until all cysteine is consumed, at which point the spectral pattern reverts back to that characteristic of γ elimination alone. The "elimination pattern" of spectra persists until all the remaining OSHS is converted to keto acid. The bases for these differences in the observed steady-state spectra are as yet unclear.

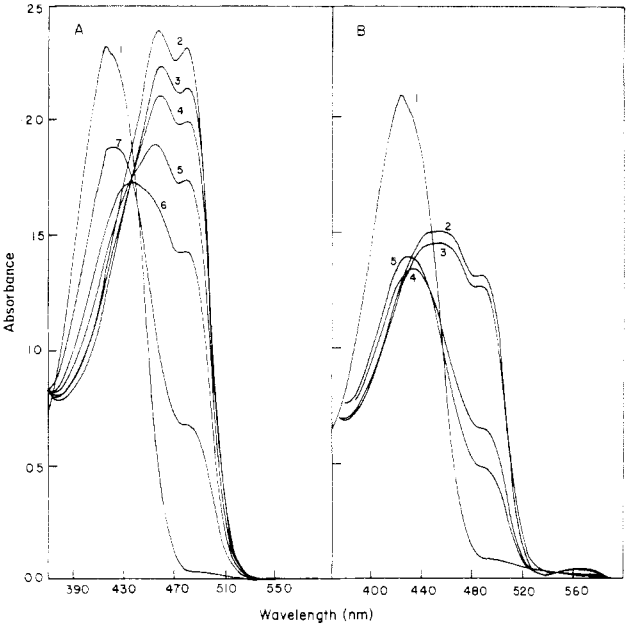


FIGURE 5: Absorbance spectra of cystathionine γ-synthetase catalyzed formation of α-ketobutyrate (β elimination) from 5 mM *erythro*-L-fluoroaminobutyrate (A) and from 5 mM *erythro*-L-chloroaminobutyrate (B). (See text for reaction conditions.) Curves A1 and B1 were obtained prior to addition of substrates. Curves A2–A7 were generated 1, 60, 65, 70, 80, and 90 min after the addition of fluoroaminobutyrate. Curves B2–B5 were generated 1, 60, 90, and 120 min after the addition of chloroaminobutyrate. The enzyme concentration was 0.95 mg/mL (5.9 nmol).

(*b*) *Vinylglycine.* The spectral data for cystathionine synthesis (reaction 1) and for ketobutyrate formation (reaction 2) with vinylglycine as substrate are given in Figure 4. It is apparent that reaction 2 for vinylglycine produces spectral changes (Figure 4A) which are quite similar to those observed for γ elimination on substrate OSHS.

For reaction 1 (Figure 4B), two spectral features are worthy of note. Addition of limiting cysteine (5 mM) to the reaction of enzyme and vinylglycine (10 mM), which is given by curve 4B2, (1) produces a nearly 50% enhancement of the 485-nm chromophore and (2) introduces a shoulder of intense absorbance between 440 and 460 nm (4B3, 4B4). As the cysteine is consumed, this plateau is lost and ultimately a spectrum characteristic of that for reaction 2 is observed (4B5).

(*c*) *Haloaminobutyrates.* Figure 5 presents the spectra for α-ketobutyrate formation (β elimination) from the two β-halo-α-amino acids, *erythro*-L-fluoroaminobutyrate (Figure 5A) and *erythro*-L-chloroaminobutyrate (Figure 5B). As can be seen from the figure, addition of the fluoroaminobutyrate to the enzyme generates large amounts of two high-wavelength chromophores, one at 460 nm and one at 485 nm. At 4 °C the spectral pattern given by curve 4A2 remains unchanged for 60 min, at which time the temperature of the reaction was raised to 25 °C to hasten the slow turnover. (A similar manipulation was required for the reaction with the chloroaminobutyrate as well.) Thus, curves 5A3–5A7 were obtained at the higher temperature. Note that there is a gradual disappearance of the two chromophores, with the 485-nm band persisting after the loss of the 460-nm peak. Indeed, the 485-nm species is evident throughout the period of recovery of the 422-nm band (note especially curves 5A6 and 5A7). These spectral changes are similar to those reported previously for β elimination on succinylserine (Guggenheim & Flavin, 1971).

For the spectra generated by the reaction of the enzyme and the chloroaminobutyrate (Figure 5B), two features are worthy

of note. The two high-wavelength chromophores which develop do not appear to accumulate in such large amounts as observed during reaction of the fluoro compound. And the second chromophore appears initially as a broad plateau of absorbance between 440 and 470 nm. Both these observations may argue for different chromophoric intermediates in the processing of the two haloamino acids. As observed for the fluoro compound, the second species dissipates prior to the loss of the 485-nm band.

It is notable that the final absorbance of the 422-nm species (5B5) is only 64% that for the enzyme prior to the addition of chloroaminobutyrate. This is a value lower than expected by dilution alone (10%) and may represent the loss of free enzyme to a covalent adduct (without 422-nm absorbance) formed by chloroaminobutyrate inactivation. The enzyme was assayed after curve 5B5 was obtained and showed only 65% its normal catalytic activity vs. a control.

We have determined the spectra for the reaction of the enzyme with chloroaminobutyrate in the presence of cysteine (data not shown). These generate patterns identical with those shown above (Figure 5B) for the processing of the haloamino acid alone, consistent with our kinetic observations, which show that the enzyme is unable to support significant thiol consumption in the presence of chloroaminobutyrate.

Discussion

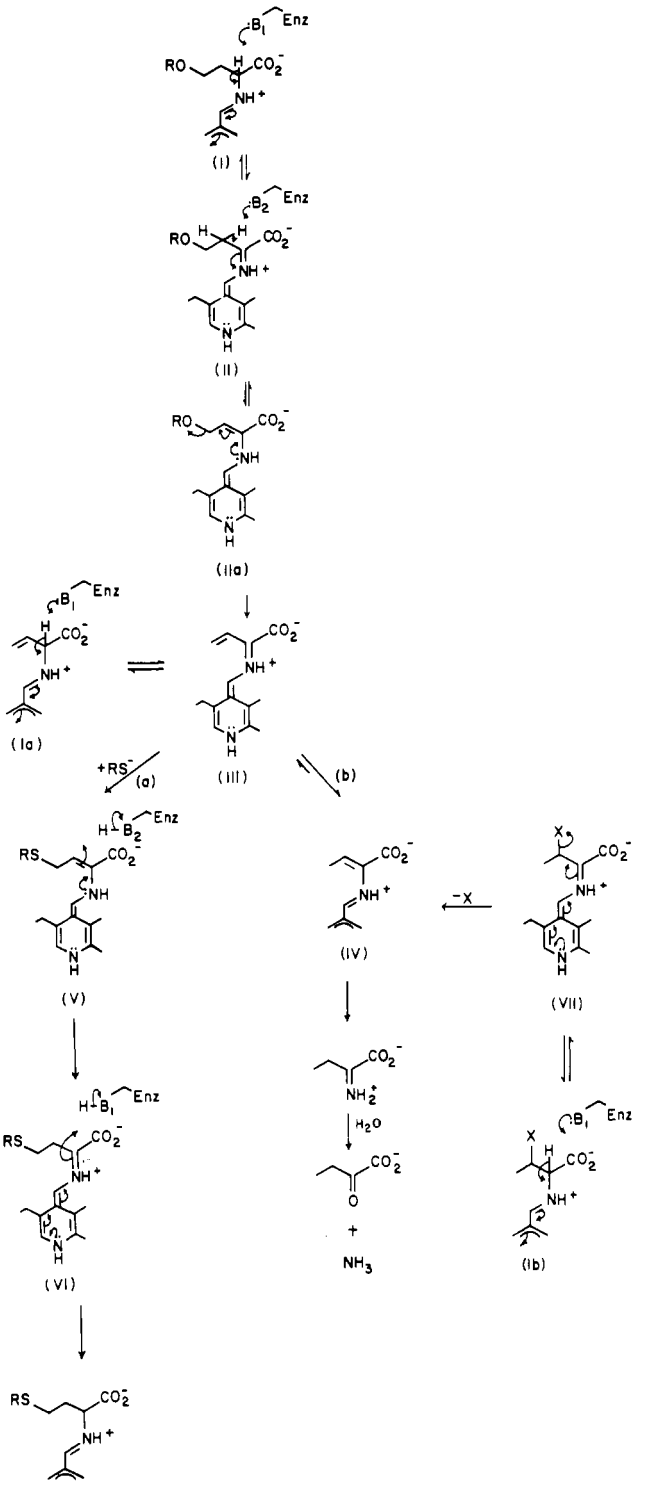
The results presented in this paper confirm our expectation that vinylglycine can enter into the normal catalytic sequence for cystathionine γ-synthetase catalyzed formation of both α-ketobutyrate or cystathionine, the products generated from the physiological substrate *O*-succinyl-L-homoserine (OSHS). The kinetic patterns for product formation from each of the two compounds are quite distinct, however. By contrast to the reactions observed with vinylglycine, the β-halo-α-aminobutyrates are processed for HX elimination to yield α-ketobutyrate but react very poorly with cysteine. The following discussion attempts to accommodate these findings, including both chemical and kinetic partitioning data for all three substrate classes, into a mechanistic scheme which features the stabilized PLP–substrate α carbanion II, the fully conjugated β,γ-unsaturated α-imino *p*-quinoid species III, and the aminocrotonate–PLP–eneamine IV, all held to be key intermediates in γ-elimination and γ-replacement sequences (Davis & Metzler, 1972).

Reactions with Haloaminobutyrates.[7] We suggest in Scheme III that the formation of α-ketobutyrate from either haloaminobutyrate (X = F or Cl) involves initially a two-step, nonconcerted halide elimination which consists of (1) a rapid abstraction of the α proton from the pyridoxaldimine adduct of the haloaminobutyrate Ib followed by (2) a slower expulsion of the β halide from the stabilized β-halo-α-carbanion VII. Elimination gives the pyridoxaldimine of aminocrotonate (IV) which ultimately suffers reverse transaldimination and hydrolysis to keto acid and ammonia. These arguments are supported by kinetic and spectral evidence.

The V_{max} values for β elimination to yield ketobutyrate are different for each of the haloaminobutyrates and may be dependent upon the differential reactivity of fluorine and chlorine to intramolecular carbanion-assisted expulsion. Thus, halide elimination is probably one of the slow steps in catalysis with these substrates. That we detect the enzyme-catalyzed incorporation of tritium into chloroaminobutyrate from tritiated water argues as well for a rapid preequilibrium α-proton exchange between solvent and a stabilized β-halo-α-carbanion (VII).

Spectral analyses (Figure 5) reveal two distinct absorbing species during the reaction of both the haloaminobutyrates, a finding which is consistent with the two-step elimination scheme proposed. It is evident from the spectra that only the 485-nm chromophore is common to reaction with both compounds; further, this species persists after disappearance

Scheme III



[7] The conclusions drawn here for the reactions of the enzyme with the two haloaminobutyrates are understood to apply as much to the reaction of β-chloroalanine, except that in the presence of the latter cystathionine γ-synthetase is able to support limited thiol consumption, presumably by β replacement (Guggenheim & Flavin, 1969a). It is possible that the steric hindrance provided by the γ-methyl substituent obviates β replacement by thiol in the case of the four-carbon haloaminobutyrates.

of the shorter wavelength chromophores. These features argue for a structure common to reaction with both chloro- and fluoroaminobutyrate which is formed *after* halide elimination. Thus we suspect that the 485-nm band could be given by the pyridoxaldimine of aminocrotonate (IV). Similarly, it is not unreasonable that the *p*-quinoidal β-halo-α-carbanion VII may have different absorbance maxima, depending upon the electronic configuration of the electronegative β substituents; and thus we suggest that the 465-nm band of Figure 5A may represent the stabilized β-fluoro-α-carbanion and that the 440–470-nm absorbance of Figure 5B could be given by the β-chloro-α-carbanion. Stabilized pyridoxal α carbanions without electronegative β substituents absorb in the 490–505-nm region (e.g., Morino & Snell, 1967; Ulevitch & Kallen, 1977).

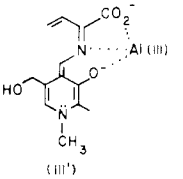
In that neither chloro- nor fluoroaminobutyrate gives kinetic evidence for significant cystathionine synthesis, we conclude that the equilibrium for tautomerization between the Michael acceptor III and the aminocrotonate pyridoxaldimine IV (incompetent for thiolate addition to carbon 4) lies far to the right, as written, and that the conversion of initially formed IV to III could be slow. By contrast, the vinylglycine data, discussed below, show that the enzyme will allow rapid conversion of species III, when formed initially, to species IV. Thus the haloaminobutyrate studies reveal that III to IV is functionally a one-way step (and must be so in vinylglycine and OSHS processing as well); once the aminocrotonate–PLP species IV has formed, the enzyme cannot convert it back to III for γ replacement.

Despite the fact that species IV will be chemically incompetent to undergo γ replacement by nucleophiles, it should undergo β replacement. The fact that V_{max} for attack by RS^- on bound aminoacrylate (from β-chloroalanine) is much faster than V_{max} for attack on IV suggests that steric hindrance by the C_4-methyl group is a serious kinetic deterrent to Michael addition.

Reactions with Vinylglycine. Vinylglycine is a substrate for turnover by cystathionine γ-synthetase and by other pyridoxal enzymes which accomplish reactions at the γ carbon of appropriate substrates (e.g., methionine γ-lyase, Esaki et al., 1977; γ-cystathionase, Washtein & Walsh, unpublished experiments) and at the β carbon of others (e.g., tryptophan synthetase, Miles, 1975; threonine deaminase, Kapke & Davis, 1975). In contrast, vinylglycine is also a suicide inactivator of varying efficiency (Walsh, 1978) for certain pyridoxal dependent transaminases, such as L-aspartate transaminase (Rando, 1974) and D-amino acid transaminase (Soper et al., 1977), which effects irreversible and covalent modification of the target protein. The mechanism of inactivation could involve nucleophilic capture of the 2-imino-3-butenoate *p*-quinoid III, the identical intermediate to which thiol addition presumably effects, not inactivation, but product synthesis in the case of γ-replacement enzymes. Thus III may be understood as an appreciably reactive electrophile which is either (1) a partitioning intermediate for the γ-replacement enzymes which bind nucleophilic cosubstrates at the olefinic terminus of III or (2) a potential killing species if enzymic nucleophiles are placed adventitiously close to the olefinic center, as may occur in the transaminases.

Therefore, given its presumed mechanistic centrality, one might expect to observe spectral evidence for the transitory accumulation of III during steady-state turnover of vinylglycine, especially since our kinetic evidence favors a rapid α-proton exchange between solvent and α-[^{3}H]vinylglycine. In the ketobutyrate forming reaction, a single high-wavelength chromophore (λ_{max} = 485 nm) is detected upon reaction with vinylglycine (Figure 4A); but we suspect that this *does not* represent accumulation of the *p*-quinoid III, according to the following arguments.

Guggenheim & Flavin (1971) suggest that the 2-imino-3-butenoate–PLP *p*-quinoid III should absorb maximally above 500 nm, consonant with the additional β,γ unsaturation of the already highly conjugated quinoid. Recently Karube & Matsushima (1977) have provided the absorbance spectrum for a model compound of structure III, an aluminum chelate of the *N*-methylpyridoxamine ketimine quinoid of 2-amino-3-butenoic acid (III′). This compound gives an absorbance band at 550 nm, and its corresponding Shiff's base aldimine absorbs at 514 nm.



(III′)

We suggest, therefore, that the stabilized α carbanion of vinylglycine does not accumulate. Certainly its formation is not rate determining, given the rate ratio of 1.72 we determine for tritium washout to solvent vs. ketobutyrate formation. The lack of any positive spectral evidence expected for a structure such as III indicates a facile prototropic isomerization of it to IV. The PLP–aminocrotonate is probably more stable than the dihydropyridine species and may represent the only accumulating absorbing intermediate between vinylglycine and keto acid. This would be the identical 485-nm species which we suspect accumulates during β-haloaminobutyrate processing; different amounts of A_{485} could reflect a different fraction of the enzyme population in this form during steady-state turnover with the different individual substrates. (The respective V_{max} values are 10–20-fold different.) The extinction coefficient for any such PLP–aminocrotonate (IV) is not known.

In the case of γ replacement upon the putative structure III, we suspect that the equilibrium favors formation of the addition product V as no absorbing species are detected above 485 nm for this reaction either (see Figure 4B). It is of interest to note that ketobutyrate formation and cystathionine synthesis from vinylglycine display essentially an identical V_{max} = 44–46 μmol min^{-1} mg^{-1}, a finding which could be consistent with a common intermediate for the two reactions, the formation of which is rate determining. However, the identity of the two V_{max} values may be fortuitous, and we suggest rather that our data argue for a minimal two-step mechanism (after the formation of III) for cystathionine synthesis. This process would involve (1) the tautomerization of a putative intermediate (V) arising from Michael addition by cysteinyl thiol, and (2) reprotonation of the stabilized α carbanion of the bound product quinoid VI.

γ replacement upon the partitioning intermediate III necessarily involves two prototropic shifts for turnover to product cystathionine. In the reaction with substrate OSHS, two substrate-derived protons are available (at least in a formal sense) at the active site from the steps which generate the α- and β-carbanion equivalents, and it may be expected (Posner & Flavin, 1972a) that these protons are returned to nascent product following capture of III by cysteine. In the case where vinylglycine is the substrate for cystathionine formation, only one substrate proton (from the α carbon) is available; and, if the enzymic base (now formally dissociated) which ordinarily provides this proton is sequestered from bulk solvent, then

either one or both of the prototropic shifts between III and product may become partially rate determining.

These arguments are supported by the kinetic observation that γ replacement on vinylglycine experiences no rate enhancement over that observed for ketobutyrate formation. By contrast, the V_{max} for γ replacement on OSHS, where the two reprotonations are not expected to be rate determining, displays a tenfold enhancement over that value for γ elimination. It may be that the V_{max} rate for replacement (but not for ketobutyrate formation) with vinylglycine as substrate is partially determined by the facility with which the enzymic base (EB_2: of Scheme III) is able to acquire protons from solvent.

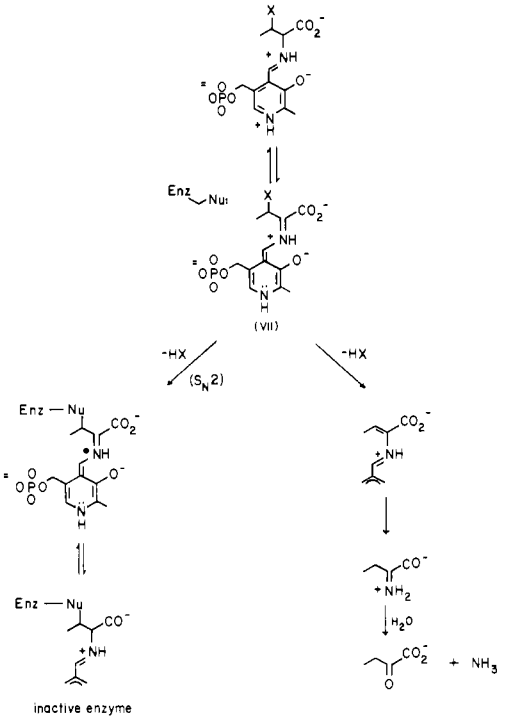
The spectral data are consistent with a two-step mechanism for replacement on III, but one makes assignment of the chromophores observed to specific structures only with difficulty. In that the 485-nm band is common to γ replacement for both reaction with OSHS (Figure 3B) and with vinylglycine (Figure 4B), this species may represent a structure like VI.[8,9] From the kinetic arguments outlined above, V might reasonably be expected to accumulate when vinylglycine experiences γ replacement, and a structure of the type may account for the absorbance plateau between 440 and 460 nm seen in Figure 4B.[9]

*Reactions with Substrate: O-Succinyl-*L*-homoserine.* Both γ elimination and γ replacement on substrate OSHS commence with the conventional proton abstractions from I and II to generate α- and β-carbanionic equivalents. It is unlikely that either I or II accumulates in the steady state since both of the proton abstractions are thought to be rapid relative to succinate elimination (Guggenheim & Flavin, 1969a,b; Posner & Flavin, 1972a).

However, we believe that the β,γ elimination is nonconcerted and—since it proceeds with a V_{max} value which is half that observed for ketobutyrate formation from vinylglycine—perhaps is at least partially rate determining. It is not inconceivable that a structure like that of IIa is a detectable intermediate, especially if elimination of HOR is slow; IIa might give rise to absorbance at 485 nm (Figure 3A). But by analogy to the ketobutyrate-forming reactions with model substrates, one might as reasonably argue that the aminocrotonate structure IV is in fact the accumulating 485-nm absorber in γ elimination on OSHS as well. The spectral data do not afford distinction between IIa and IV.[10]

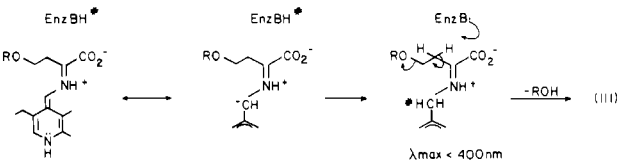
Our data for γ replacement on the substrate agree with the conventional hypothesis, as outlined in Scheme III, where the observed high-wavelength absorbing species (Figure 3B) could arise either from IIa or VI. It seems unlikely, however, that VI could be an appreciable contributor to the 485-nm absorbance observed for replacement on OSHS. By contrast to the corresponding reaction for vinylglycine, the two tautomerizations (V to VI and VI to product-bound pyridoxaldimine) are suspected to be facile. Some of the 485-nm absorbance is possibly contributed by IV, since partitioning between path a and path b is expected when the cysteine concentration is limiting.[11]

Scheme IV



Inactivation by Haloaminobutyrates. The ability of the two haloaminobutyrates to serve both as substrates for ketobutyrate formation and to effect inactivation of cystathionine γ-synthetase would seem to arise by partitioning from a common intermediate (VII), the stabilized α-halo-β-carbanion (Scheme IV). Structure VII as, at least in part, a β-haloimine species may suffer ready S_N2 displacement of the halide at carbon 3 by an enzyme nucleophile. Reprotonation of the α carbon would generate a stable, covalently labeled enzyme. We

originally proposed a scheme of this sort as consistent with a single polyhydric base acting to generate *both* α- and β-carbanion equivalents; they suggested that the base might be the ε-amino function of an active-site lysyl residue. In this event the PNP-oxidation state cannot persist but would have to be quantitatively returned to the PLP-oxidation state for processing of III and product release.



[8] Some of this absorbance, especially late in the reaction, might also be given by IV as the substrates partition between path a and path b as cysteine is consumed.

[9] Steady-state spectroscopic analyses are insufficient, of course, for the unambiguous assignment of chemical structure. Compounds V and VI, for example, may be quite similar in spectroscopic character, with values for maximal absorbance and extinction coefficients which are relatively close; the exact values are unknown. Moreover, single-turnover, stopped-flow experiments are necessary for the identification of the number of intermediates obtained with each of these substrates and for an evaluation of their temporal sequence.

[10] For the reactions with the physiological substrate, where a potentially complex set of structural rearrangements may obtain, the accurate correlation of a small number of detectable chromophores with a large number of chemically rational intermediates is especially difficult, if not impossible, from steady-state spectral analyses alone. The assignment of absorbing species to specific structures is of necessity informed by analogy to the model reactions, depends to a great extent upon chemical reasoning, and remains—at best—tentative.

[11] A curious aspect of the steady-state spectra obtained for reaction of OSHS (especially by contrast with those for reaction of the haloaminobutyrates) is that bleaching of the 422-nm chromophore apparently is not accompanied by a quantitatively correspondent accumulation of the 485-nm species, as though much of the enzyme were present as a "silent" chromophore, i.e., $\lambda_{max} < 400$ nm. (In the case of the haloaminobutyrates, the 422-nm band appears to be replaced quantitatively by the 485-nm chromophore in the steady state.) This might conceivably suggest *fully reversible* formation of enzyme–pyridoxamine-P species from OSHS. This substrate is distinct in requiring accommodation of two substrate-derived protons (α and β) in the active site; that is, two base equivalents are necessary for catalysis. One base could be an enzyme amino acid side chain; the other could conceivably be the benzylic carbon of the pyridoxal cofactor in its *p*-quinoid resonance form. Posner & Flavin (1972b)

observe that neither vinylglycine nor OSHS gives any inactivation, which further directs attention to structure VII as the likely inactivating species, rather than other potential conjugated electrophiles common to those two amino acid substrates.

It is also of interest to note that the rate constant for partial inactivation by chloroaminobutyrate (5.8×10^{-4} s^{-1}) is larger than that for inactivation by fluoroaminobutyrate (2.8×10^{-4} s^{-1}). Insofar as these values express a difference in the rate for halide elimination in a rate-determining step, they confirm a mechanism of inactivation by nucleophilic displacement wherein chlorine is expected to be a better leaving group than fluorine. By contrast, α-carbanion-assisted elimination of the halide, to give eventual formation of α-ketobutyrate, appears to favor fluoride over chloride as a leaving group (Table I). These rate differences, both for inactivation and for processing of the haloamino acids to product ketobutyrate, point to the importance of the stabilized α-halo-β-carbanion VII as a focal kinetic intermediate in both catalysis and inactivation.

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Affinity Labeling of Catalytic and Regulatory Sites of Pig Heart Mitochondrial F_1-ATPase by 5′-*p*-Fluorosulfonylbenzoyladenosine†

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ABSTRACT: Pig heart mitochondrial F_1-ATPase is inactivated on incubation with the adenine nucleotide analogue 5′-*p*-fluorosulfonylbenzoyladenosine (FSBA). The inhibition is reversible if the incubation time does not exceed 1 min and depends on the ATP concentration. The Hill number (h = 1.8 ± 0.2) indicates cooperative binding of FSBA. After 1 min of incubation, FSBA produces irreversible inactivation characterized by biphasic kinetics. The initial, rapid phase diminishes the maximal velocity of the enzyme without modifying the K_m for ATP. The pseudo-first-order rate constant of the rapid phase is independent of FSBA concentration between 0.09 and 0.57 mM, indicating a very high affinity for the analogue. In contrast, the pseudo-first-order rate constant for the second, slower phase depends on FSBA concentration with saturating kinetics; this indicates that a reversible enzyme–inhibitor complex is formed before the irreversible reaction, with a K_D (0.23 mM) similar to the K_m for ATP (0.26 mM). The relationship between the incorporation of [*benzoyl*-^{14}C]FSBA during inactivation and the residual activity of the enzyme is also biphasic and gives an extrapolated value of 6 mol bound per mol of enzyme for complete inhibition. A value of 1–4 mol of FSBA bound per mol of enzyme is calculated for the rapid phase and a maximal number of 2 mol bound for the slow phase. The rapid phase of inactivation appears to reflect the binding to regulatory sites, whereas the slow phase appears to correspond to the binding to catalytic sites.

The purpose of this work was to label irreversibly the regulatory and catalytic sites of F_1-ATPase with an analogue of ATP and ADP. The use of such an analogue with an unmodified adenosine moiety is essential for the understanding of catalytic and regulatory mechanisms of the enzyme. It is generally recognized that F_1-ATPase plays a central role in ATP synthesis when integrated in the mitochondrial membrane, this process being very specific for the adenosine moiety. The presence of regulatory sites, able to bind ADP or ATP, has been demonstrated both by the detection of cooperativity in kinetic studies (Godinot et al., 1975; Ebel & Lardy, 1975;

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