

Mechanistic Studies on Reactions of Bacterial Methionine γ -Lyase with Olefinic Amino Acids[†]

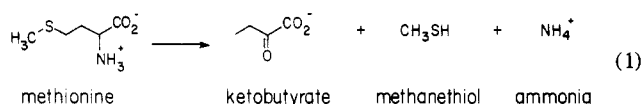
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ABSTRACT: Methionine γ -lyase (EC 4.4.1.11), which catalyzes the formation of methanethiol, α -ketobutyrate, and ammonia from L-methionine (eq 1), promotes the oxidative deamination of several four- and five-carbon olefinic amino acids (1-5). With the exception of vinylglycine (1), the V_{\max} rates of keto acid formation from the unsaturated substrate analogues are substantially lower than that for processing of methionine to α -ketobutyrate; vinylglycine is deaminated to ketobutyrate and ammonia with a V_{\max} twice that for L-methionine turnover. L-Allylglycine, L-2-amino-3-*trans*-pentenoate, and L-2-amino-3-*cis*-pentenoate (2, 4, 5) are all converted to 2-ketopentanoic acid (α -ketovalerate). L-2-Amino-3-*cis*-pentenoate (5) is also a time-dependent, irreversible inactivator of the enzyme. None of the other substrate analogues tested appears to inactivate the enzyme. Spectral analysis of the enzymatic

reaction with *cis* isomer 5 reveals the formation of a high-wavelength chromophore ($\lambda_{\max} = 550$ nm) which implies that a β,γ -unsaturated pyridoxal *p*-quinoid (VI) accumulates. No such absorbing species appears to form during the reaction of *trans* isomer 4 with methionine γ -lyase. But a 550-nm chromophore develops when both 4 and 5 are reacted with $\text{Al}(\text{NO}_3)_3$ and pyridoxal methochloride in methanolic KOH. It would appear that the geometry of the protein and the olefinic amino acid as an intermediate enzyme-substrate adduct controls the kinetics of reaction, such that azaallylic isomerization becomes selectively rate determining for reaction with 5. When this isomerization is slow, an accumulating Michael-type acceptor (VI) could lead to the observed irreversible inactivation of the enzyme.

Microbial methionine γ -lyase (EC 4.4.1.11) catalyzes the formation of α -ketobutyrate, methanethiol, and ammonia from the substrate L-methionine (eq 1). The enzyme has been purified to homogeneity by Soda and his colleagues (Tanaka et al., 1977) and has been shown to be an $\alpha_2\beta_2$ tetramer of four subunits of identical molecular size (mol wt 45 000) but nonidentical in molecular charge (Johnston et al., 1979b). Each subunit contains one residue of tightly bound pyridoxal 5-phosphate (PLP).¹

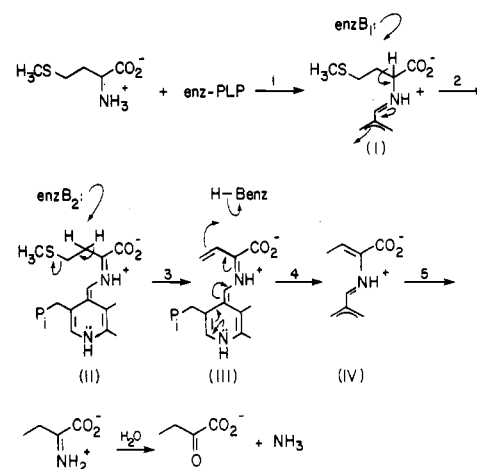
The key mechanistic features which account for the oxidative deamination of methionine (eq 1) include pyridoxal-



assisted γ elimination followed by a net 1,4-azaallylic isomerization (Scheme I). Substrate methionine undergoes (1) the usual transaldimination with enzyme-pyridoxal, (2) α -proton abstraction to yield an α -carbanion equivalent stabilized as a pyridoxalimine *p*-quinoid, and (3) β -proton removal, which leads to the elimination of the γ -thiomethyl group and formation of a β,γ -unsaturated *p*-quinoid (III).

Conversion of III to IV (the PLP-aminocrotonate derivative) constitutes the azaallylic isomerization step; then reverse

Scheme I



transaldimination (step 5) forms iminobutyrate and regenerates the cofactor poised for the next catalytic turnover. Hydrolysis to ketobutyrate presumably occurs off the enzyme.

Structure III is thought to be a key partitioning intermediate which forms during the reaction of all enzymes catalyzing γ eliminations and γ replacements. It may undergo nucleophilic addition at the C₄ vinyl carbon (γ replacement), as in the biosynthesis of cystathionine promoted by cystathionine γ -synthetase (Davis & Metzler, 1972; Johnston et al., 1979a), or it may isomerize prototropically to the enamino pyridoxalimine (IV) in the absence of an adding nucleophile, as is suggested for methionine γ -lyase above and for γ -cystathionase (γ elimination; Davis & Metzler, 1972). Direct experimental evidence for the formation of a β,γ -unsaturated pyridoxal *p*-quinoid (III) has been obtained to date only in model organic

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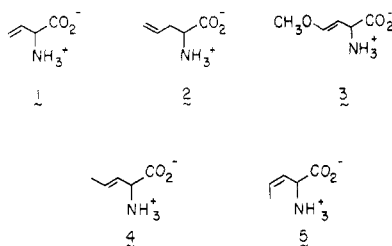
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¹ Abbreviations used: NADH, reduced nicotinamide adenine dinucleotide; KP_i, potassium inorganic phosphate; KPP_i, potassium inorganic pyrophosphate; LDH, lactate dehydrogenase; PLP, pyridoxal 5-phosphate; PNP, pyridoxamine 5-phosphate; TLC, thin-layer chromatography.

reactions (Karube & Matsushima, 1977); in enzymatic systems, its formation as a kinetically competent intermediate has been only inferred. To search for direct evidence for intermediate III, we have reasoned that each of the olefinic amino acids 1–5 should be substrates for the azaallylic isomerization (III \rightarrow IV).



Enzymatic processing of olefinic amino acids in pyridoxal-catalyzed reactions is of mechanistic interest for several reasons. Note, for example, that the partitioning intermediate III is the PLP-stabilized α carbanion of vinylglycine (1). Thus vinylglycine may be expected to be a substrate for all pyridoxal enzymes which catalyze γ elimination and replacement sequences, and, indeed, we have previously shown that 1 is rapidly converted to α -ketobutyrate and ammonia by cystathionine γ -synthetase (Johnston et al., 1979a) and by methionine γ -lyase (Esaki et al., 1977).

Compounds 1–3 are natural products (Scannell et al., 1971), and each is a known inactivator of certain pyridoxal-dependent enzymes. Vinylglycine (1), for example, inactivates both L-aspartate transaminase (Rando, 1974a) and D-amino acid transaminase (Soper et al., 1977). D-Allylglycine is a powerful convulsant, first oxidized by a brain D-amino acid oxidase (Walsh et al., 1978a) and then giving rise to inhibition of the PLP-dependent brain glutamate decarboxylase (Orlowski et al., 1977).

Substituted amino acid vinyl ethers (such as 3 and rhizobitoxine) are known inhibitors of PLP-dependent enzymes. The *trans*-methoxy-2-buten-1-olate (3) inactivates aspartate aminotransferase (Rando, 1974b) and tryptophan synthase (Miles, 1975). Rhizobitoxine is reported to inactivate β -cystathionase from plants and thereby prevents biosynthesis of the ripening hormone ethylene (Giovaneli et al., 1971). Compounds 4 and 5 are synthetic geometric isomers of a β,γ -olefinic amino acid not previously characterized as substrates for any pyridoxal enzyme.

We report here kinetic and spectroscopic data for the processing of 1–5 by methionine γ -lyase and show that each of these compounds is a substrate. Further we show that 5 allows detection of a β,γ -unsaturated *p*-quinoid–enzyme adduct, to our knowledge inferred, but not seen previously in a reaction catalyzed by a γ -elimination enzyme. Time-dependent inactivation of methionine γ -lyase by 5 is also described.

Materials and Methods

Enzyme Purification. Methionine γ -lyase (EC 4.4.1.11) was purified from *Pseudomonas ovalis* (IFO 3738) according to the methods of Tanaka et al. (1977). The specific activity with substrate methionine (eq 1, assay described below) was 3.2 units/mg. Holoenzyme used in these experiments had absorbance maxima at 280 and 418 nm; the A_{280}/A_{418} was 4.7.

We have reported previously (Johnston et al., 1980) that methionine γ -lyase is unstable to storage for long periods. However, we have now determined that the specific activity (and the A_{280}/A_{418} ratio) of freshly prepared enzyme can be maintained for up to 6 months if the enzyme is stored at -20°C in 20 mM KPi buffer containing 50 μM PLP and 1.0 mM

dithiothreitol. Beyond 6 months, the enzyme loses activity, but we have been successful in recovering native specific activity when these “aged” samples are dialyzed against fresh solutions of the storage buffer. It appears that long-term instability results from both slow sulphydryl autooxidation and dissociation of the pyridoxal cofactor.

Substrates. L-Methionine was purchased from Aldrich and was used without further purification. L-Allylglycine (2-amino-4-pentenoate) and α -ketovalerate (2-ketopentanoate) were Sigma products. DL-Vinylglycine (2-amino-3-buten-1-olate) was prepared according to the methods of Baldwin et al. (1977) and was then resolved by the treatment of *N*-chloroacetyl-DL-vinylglycine with hog kidney acylase I, purchased from Sigma. 2-Amino-4-methoxy-3-*trans*-butenoate (Scannell et al., 1971) was the generous gift of Dr. Ronald Kaback, Roche Institute of Molecular Biology.

2-Amino-3-*cis*-pentenoate was prepared in the following way. 2-Butynaldehyde diethyl acetal (14.2 g, 0.1 mol) was stirred at room temperature with 200 mL of a 1:1:1 mixture of dioxane, acetic acid, and water for 48 h. To the light yellow solution was added KCN (7.8 g, 0.12 mol) in one portion, and the mixture was stirred for 24 h. Concentrated hydrochloric acid (75 mL) was then added, and the mixture was heated on a steam bath for 7 h. It was cooled to room temperature and concentrated to a dark brown syrupy residue. This was dissolved in 200 mL of water. The pH was adjusted to 8.5 with solid Li_2CO_3 , and the solution was washed 3 times with 50-mL portions of CH_2Cl_2 , which removed most of the color to the organic layer. The aqueous layer was taken and the water was removed in vacuo to give a white solid of 2-hydroxy-3-pentynoate lithium salt, weighing 10.0 g (70% yield) after drying.

To the above lithium salt, 100 mL of 1 N HCl was added. The mixture was continuously extracted with 200 mL of ether for 24 h. Freshly prepared diazomethane was added to the ether extract until a bright yellow color persisted. Nitrogen was bubbled through the solution for 5 min to get rid of excess diazomethane, and the ether was removed in vacuo. The product methyl ester of 2-hydroxy-3-pentynoate was distilled at 63°C (1 mm), giving a colorless oil weighing 9.3 g. The synthesis of this methyl ester has also been reported by Cromartie et al. (1974).

To 9.0 g of the above methyl ester in 100 mL of acetone was added 2 mL of quinoline (freshly distilled from Zn dust) and 300 mg of Lindlar catalyst (Marcotte & Walsh, 1978). The mixture was hydrogenated at room temperature and 50 psi of hydrogen pressure. The catalyst was filtered off and acetone was removed in vacuo. Methanol (50 mL) and dry Dowex 50 cation-exchange resin (10 g, 50–100 mesh, H^+ form) were added to the mixture which was stirred at room temperature for 2 h to remove quinoline. The product methyl ester of 2-hydroxy-3-*cis*-pentenoate was fractionally distilled at 43°C (1 mm); 6.0 g was obtained. Also recovered was 2.5 g of starting material which distilled at 63°C (1 mm).

2-Hydroxy-3-*cis*-pentenoate methyl ester (6.0 g, 48 mmol) was stirred with 60 mL of 1 N NaOH at 0°C for 30 min and then at room temperature for 60 min; the solution turned slightly cloudy. Charcoal (1.0 g) was added and the solution heated briefly and filtered. The filtered solution was concentrated to 20 mL and the pH adjusted to 1.0 with 6 N HCl. Continuous extraction was carried out with 200 mL of ether for 24 h. The ether extract was dried and concentrated to 50 mL and then cooled in an ice bath as 2.8 mL of PBr_3 (freshly distilled) in 20 mL of dry ether was added slowly over a period of 20 min. The resulting mixture was then allowed to warm to room temperature and was stirred for 18 h. The reaction

was stopped by addition of 15 mL of water at 0 °C, and the mixture was stirred for 30 min. Layers separated and the ether layer was dried and concentrated to a yellow oil; 15 mL of water was added, and vigorous stirring resulted in a fine suspension. To this was added 200 mL of $(\text{NH}_4)_2\text{CO}_3/\text{NH}_4\text{OH}$ buffer solution at 0 °C. The mixture was stirred at 0–3 °C for 24 h and then at room temperature for 72 h. The solvent was removed in vacuo to give a yellow semisolid which was dissolved in 15 mL of water and loaded on a 200-mL Dowex 50 cation-exchange column (50–100 mesh, H^+ form). The column was washed with water until a pH of 7 was obtained and then eluted with 1 N NH_4OH ; 5-mL fractions were collected. Ninhydrin-positive fractions were combined and concentrated to give a light yellow crystalline material which was recrystallized from 60% aqueous ethanol to give 1.5 g of white crystalline 2-amino-3-*cis*-pentenoic acid (27% yield from the methyl ester): mp 240 °C dec; NMR (60 MHz) δ 1.6 (3 H, dd, $J = 7$ and 2 Hz), 4.5 (1 H, d, $J = 10$ Hz), 5.0–6.0 (2 H, m). Anal. Calcd for $\text{C}_5\text{H}_9\text{NO}_2$: C, 52.15; H, 7.89; N, 12.17; O, 27.79. Found: C, 51.96; H, 8.04; N, 11.97; O, 27.89.

2-Amino-3-*trans*-pentenoate was prepared by hydrolysis of crotonaldehyde cyanohydrin, as described by Rossi & Schinz (1948). Crotonaldehyde (8.4 g, 0.10 mol) and KCN (7.8 g, 0.12 mol) were reacted as outlined for the *cis* compound: yield, 8.1 g of 2-hydroxy-3-*trans*-pentenoate (70%).

2-Hydroxy-3-*trans*-pentenoate (5.8 g, 0.05 mol) was reacted with $\text{PBr}/(\text{NH}_4)_2\text{CO}_3/\text{NH}_4\text{OH}$ by the same procedure described above to give 1.65 g of crystalline 2-amino-3-*trans*-pentenoate (30% yield): mp 245 °C dec; NMR (60 MHz) δ 1.7 (3 H, d, $J = 5$ Hz), 4.2 (1 H, d, $J = 8$ Hz), 5.2–6.2 (2 H, m). Anal. Calcd for $\text{C}_5\text{H}_9\text{NO}_2$: C, 52.15; H, 7.89; N, 12.17; O, 27.79. Found: C, 52.23; H, 7.92; N, 12.14; O, 27.62. Similar data for elemental analysis have been given for the *trans* compound by Marcotte & Walsh (1978).

The *cis* and *trans* isomers of DL-2-amino-3-[2- ^3H]pentenoate were prepared by using arginine racemase partially purified according to the methods of Yorifugi et al. (1971a, b). Reactions were carried out in the following way. To a solution of 1 mL of 10 mM KPi buffer, pH 7.3, were added 23 mg (~ 200 μmol) of either L-2-amino-3-*cis*- or -*trans*-pentenoate, 6.0 units of racemase (~ 10 units/mg), and 200 μL of $^3\text{H}_2\text{O}$ (1 Ci/mL). The reaction was incubated for 5 h at 37 °C. 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 tritium levels fell to background and was then eluted with 2 N NH_4OH . The fractions containing radioactivity were pooled, neutralized, and lyophilized 3 times. The lyophilized residues (obtained from reaction of either *cis*- or *trans*-pentenoate) gave a single radioactive ninhydrin spot which cochromatographed (butanol/acetic acid/water, 4:1:1) with authentic 2-amino-3-pentenoate. This procedure gave 2-amino-3-*trans*-[2- ^3H]pentenoate of specific activity 900 cpm/nmol and 2-amino-3-*cis*-[2- ^3H]pentenoate of ~ 210 cpm/nmol.

Pyridoxal methochloride was prepared by slight modification of the procedures of Heyl et al. (1951). Best results were obtained when the intermediate pyridoxal methiodide monomethyl acetal, prepared by the Heyl synthesis, was treated with freshly prepared silver chloride and the aqueous reaction carried out in the dark. After filtration, the water was evaporated to dryness, and a solid yellow product was obtained. This residue was dissolved in water and titrated to pH 1.5 with 1 N HCl to hydrolyze the acetal. The solution was then decolorized with charcoal, filtered, and evaporated in vacuo. A yellow oil was obtained which gave crystallization from

water/acetone. The yield was ~ 500 mg (76%). The product had a major absorbance band with $\lambda_{\text{max}} = 328$ nm in 10^{-3} M KOH in methanol. Professor David Metzler kindly provided us with a small sample of pyridoxal methochloride to use as seed crystals. Our synthetic sample gave the same UV absorbance band and a 60-MHz proton spectrum identical with that of the authentic material.

Kinetic Assays. Formation of keto acid products from substrate methionine and from the substrate analogues was followed by reduction of product to the hydroxy acid in the presence of NADH and lactate dehydrogenase (Boehringer-Mannheim, from rabbit skeletal muscle). The assay conditions have been described (Johnston et al., 1979b).

The rate of enzyme-catalyzed proton exchange between 2-amino-3-[2- ^3H]pentenoate (both *cis* and *trans* isomers) and solvent water was determined in the following way. A 1.5-mL solution was prepared containing 50 mM KPi buffer (pH 8.3, 37 °C), 0.6 mM NADH, 1.0 mg of LDH, and either 25 mM 2-amino-3-*trans*-[2- ^3H]pentenoate (900 cpm/nmol) or 125 mM 2-amino-3-*cis*-[2- ^3H]pentenoate (200 cpm/nmol). The reaction was initiated by addition of 20 μg of methionine γ -lyase. Ketobutyrate formation was followed by disappearance of absorbance at 340 nm. At timed intervals, 100- μL aliquots were removed from the incubation mixture and loaded onto a Dowex 50 H^+ column. Each column was eluted with 2.0 mL of water, and the water was counted in 15.0 mL of ACS scintillant. This procedure allowed the simultaneous determination in a single assay of the rates of both ketobutyrate formation and of tritium "washout to solvent".

Inactivation Kinetics. Inactivation experiments involved the following protocol. A 90- μL solution containing an appropriate concentration of a substrate analogue was prepared in 50 mM KPi buffer, pH 8.3, at 37 °C. At time zero, 10 μL of methionine γ -lyase (13.5 mg/mL) was added. At timed intervals, 10- μL aliquots were removed and assayed by dilution to cuvettes containing 1 mL of 25 mM NADH, 0.3 mg of LDH, and saturating methionine (50 mM). Ketobutyrate formation was followed by loss of absorbance at 340 nm.

Spectral Studies. Steady-state absorbance spectra were obtained with a Perkin-Elmer 554 scanning spectrophotometer. A typical spectral analysis of enzyme and substrate involved the following protocol. An initial absorbance scan was made on a solution of methionine γ -lyase (usually 250–500 μg) in KPi buffer containing 2-mercaptoethanol or dithiothreitol. At time zero, 50–100 μL of substrate was added, bringing the total volume to 500 μL , the buffer concentration to 50 mM, pH 8.3, and the thiol concentration to 1 mM. The enzyme concentration in individual experiments was thus between 0.25 and 0.5 mg/mL and the substrate saturating (the concentration of which was dictated by the K_M value determined from kinetic analysis). Scans were made repetitively following substrate addition.

Spectral analyses of pyridoxal methochloride/aluminum-catalyzed deamination of methionine, vinylglycine, and of both the *cis* and *trans* isomers of 2-amino-3-pentenoate were carried out according to the methods of Karube & Matsushima (1977). A typical spectral determination was carried out on a methanol solution of 0.1 mM pyridoxal methochloride containing 0.2 mM KOH, 100 mM amino acid, and 0.1 mM $\text{Al}(\text{NO}_3)_3$.

Product Formation. Keto acid products were identified by thin-layer chromatography. A typical 1-mL reaction mixture contained 100 mM amino acid and 250 μg of methionine γ -lyase in 100 mM KPi buffer, pH 8.3, 37 °C. After incubation for 24 h, the solution was loaded onto a Dowex 50

Table I: Kinetic Parameters for Keto Acid Production Catalyzed by Methionine γ -Lyase

substrate	K_M (mM)	V_{max} ($\mu\text{mol min}^{-1} \text{mg}^{-1}$)
L-methionine	2.3	3.2
L-vinylglycine (1)	23	6.4
L-allylglycine (2)	0.35	0.36
L-2-amino-4-methoxy-3- <i>trans</i> -butenoate (3)	2.0	0.06
L-2-amino-3- <i>trans</i> -pentenoate (4)	19.9	1.1
L-2-amino-3- <i>cis</i> -pentenoate (5)	28.9	0.07

H^+ column and the column eluted with 5 mL of H_2O . The eluent was lyophilized to dryness, dissolved in a small volume (50–100 μL) of water and spotted onto 0.1-mm cellulose thin-layer sheets. The chromatograms were run in butanol/acetic acid/water (4:1:1) and were visualized by spraying with aniline/xylose.

Keto acid products formed in the pyridoxal methochloride/aluminum-catalyzed oxidation of the *cis* and *trans* isomers of 2-amino-3-pentenoate were also identified by TLC. The reaction mixture contained 1 mM pyridoxal methochloride, 2 mM KOH, 1 mM $\text{Al}(\text{NO}_3)_3$, and 100 mM amino acid, all in methanol. The reaction was incubated at room temperature for 48 h, after which the solvent was evaporated in vacuo. The residue was dissolved in 0.1 N HCl and extracted into ether. The ether extract was evacuated and the residue dissolved in water. The aqueous extract was then spotted onto 0.1-mm cellulose thin-layer plates, together with samples of authentic 2-ketopentanoate (α -ketovalerate). The chromatograms were run in either butanol/acetic acid/water (4:1:1) or butanol/ethanol/ammonia (7:1:2) and were visualized by spraying with aniline/xylose.

Results

Product Identification and Kinetics. Methionine γ -lyase will catalyze the formation of NADH/LDH-reducible products from substrate methionine and from the substrate analogues 1–5. For methionine and vinylglycine (1), the products are α -ketobutyrate and ammonia (Esaki et al., 1977). For the five-carbon amino acids allylglycine (2) and the two geometric isomers (*cis* and *trans*) of 2-amino-3-pentenoate (4 and 5), the product is α -ketovalerate, as evidenced by thin-layer chromatography. Thin-layer sheets showed a single aniline/xylose reactive spot cochromatographing with authentic α -ketovalerate (R_f 0.42; butanol/acetic acid/water, 4:1:1) when 2, 4, or 5 was reacted with enzyme.²

We assume that the reaction product formed from 3 is the 2-keto-4-methoxybutyrate. Chromatographic results indicate a single product with R_f = 0.55 in butanol/acetic acid/water (4:1:1); the *trans*-methoxy olefinic amino acid has an R_f of 0.08 in the same solvent system.

Table I gives the kinetic data obtained for keto acid formation with each of the amino acid substrates. The data for L-methionine are identical with those previously reported (Johnston et al., 1979b). Soda has reported (Esaki et al., 1977) a K_M value of 1.3 mM and a V_{max} of 1.4 units/mg for methionine as the substrate; a K_M for vinylglycine of 15 mM and a V_{max} of 1.8 units/mg are also reported. These data were obtained by using a discontinuous assay which detected the hydrazone derivative of keto acid product (Soda, 1968).

² The chromatographic result does not rule out the unlikely possibility of formation of a 2-keto-4-pentenoate from allylglycine.

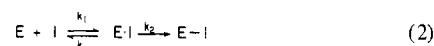
Table II: Rate Data for Reactions of Methionine γ -Lyase^a with DL-2-Amino-3-[2-³H]pentenoates

substrate	rate of product formation (nmol min ⁻¹)		rate ratio, $v([\text{P}^3\text{H}]\text{H}_2\text{O})/$ $v(\text{KV})^b$
	α -keto- valerate	$[\text{P}^3\text{H}]\text{-}$ H_2O	
DL-2-amino-3- <i>trans</i> -[2- ³ H]- pentenoate (25 mM)	1.2	5.0	4.17
DL-2-amino-3- <i>cis</i> -[2- ³ H]- pentenoate (125 mM)	2.9	9.9	3.45

^a 20 μg of enzyme in 50 mM KPP_i buffer, pH 8.3, 37 °C. Other reaction conditions are given in the text. ^b KV = ketovalerate.

Table I shows that there is considerable variation both in the binding and in the rate of turnover of the various olefinic substrates. Of particular note are the rate differences for the two 2-amino-3-pentenoates. While both 4 and 5 give apparent binding constants (K_M) of the same order, the *cis* compound is processed at a rate which is only 6.5% that of the corresponding *trans* isomer. Similarly, the *trans*-methoxybutenoate has a V_{max} nearly 20-fold lower than that of the *trans*-methyl compound. The insertion of a single oxygen atom has such a retarding effect that its influence may be electronic (at some intermediate step) rather than steric.

Inactivation Kinetics. L-2-Amino-3-*cis*-pentenoate (5) is both a substrate and a time-dependent, irreversible inactivator of methionine γ -lyase. The inactivation kinetics are pseudo-first-order, conforming to the reaction scheme outline in eq 2



where E·I represents a noncovalent enzyme inhibitor complex and E–I is a covalently modified, irreversibly inactivated enzyme (Walsh et al., 1978b). The kinetic parameters which describe eq 2 are the dissociation constant for the saturable binding step ($K_I = k_{-1}/k_1$) and the overall first-order rate constant (k_2), which describes the rate of inactivation at infinite inhibitor concentration. For inactivation of methionine γ -lyase by 2-amino-3-*cis*-pentenoate, $K_I = 40$ mM (compare $K_M \approx 30$ mM) and $k_2 = 0.07 \text{ min}^{-1}$. The k_{cat} value for processing of 5 to α -ketovalerate (calculated from the V_{max} value of Table I and based upon a mol wt of 45 000 daltons/enzyme monomer) is 3.2 min^{-1} . The ratio k_{cat}/k_2 describes the partitioning between the turnover of 5 to keto acid product vs. the number of inactivation events; the value $k_{cat}/k_2 = 45.6$. Thus, inactivation of methionine γ -lyase by 5 occurs once in roughly 50 turnovers.

Enzyme which has been fully inactivated by 5 is not reactivated by prolonged dialysis, suggesting that the enzyme becomes covalently modified. Neither the *trans*-amino-pentenoate (4) nor any of the other substrate analogues tested give detectable inactivation of methionine γ -lyase during turnover. Thus the *cis*-olefin geometry is specifically problematic to this enzyme.

Tritium "Washout" Experiments. We have determined the rate of enzyme-catalyzed tritium washout to solvent water compared to the rate of ketovalerate formation by using both DL-2-amino-3-*trans*-[2-³H]pentenoate and DL-2-amino-3-*cis*-[2-³H]pentenoate. These data are shown in Table II. For both compounds tritium is washed out to solvent at rates at least 3–4-fold (uncorrected for any kinetic isotope selection) greater than those of product ketovalerate formation. These results suggest that α -proton removal from either geometric isomer is not a rate-determining step in catalysis.

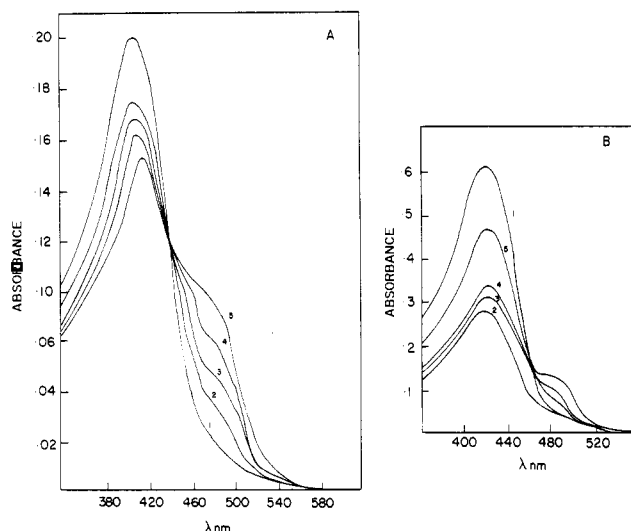


FIGURE 1: (A) Absorbance spectra obtained during methionine γ -lyase catalyzed formation of α -ketobutyrate from substrate L-methionine. Curve 1 was obtained from a 375- μ L solution containing 400 μ g of enzyme, prior to the addition of substrate. Curves 2–5 were obtained 5, 10, 20, and 30 min after the addition of 125 μ L of 100 mM L-methionine (final concentration 25 mM) (50 mM KPP_i, pH 8.3, 20 °C). (B) Absorbance spectra obtained during methionine γ -lyase catalyzed formation of α -ketovalerate from allylglycine (2). Curve 1 was obtained from a 450- μ L solution containing 1.1 mg of enzyme, prior to the addition of substrate. Curves 2–5 were obtained 1, 2, 5, and 10 min after addition of 50 μ L of 50 mM L-allylglycine (final concentration 10 mM) (50 mM KPP_i, pH 8.3, 25 °C).

Spectral Studies of Enzymatic Reaction. The steady-state absorbance spectrum obtained during turnover of L-methionine to α -ketobutyrate, catalyzed by methionine γ -lyase (Figure 1A), is characterized by the development of a single absorbing species which appears to plateau in the range of 480–500 nm. Accumulation of the high-wavelength chromophore is paralleled by the loss of the pyridoxalimine absorbance at 418 nm. There is a single isosbestic point at 440 nm. Once the reaction has gone to completion, the 480–500-nm band is lost and the 418 nm absorbance is recovered. Similar spectral events have been reported for the steady-state turnover of *O*-succinyl-L-homoserine to ketobutyrate (γ elimination) by cystathionine γ -synthetase (Guggenheim & Flavin, 1971; Johnston et al., 1979a).

Spectral observations made for the enzymatic processing of vinylglycine (1) are qualitatively³ identical with those made for turnover of methionine (data not shown). There is a single chromophore at 485 nm developing after the addition of vinylglycine which is accompanied by the loss of the 418-nm band. The two species are isosbestic at 440 nm. Thus, Figure 1A serves also to describe the spectral events for enzymatic action on 1.

Figure 1B gives the spectral events which describe the enzymatic turnover of L-allylglycine (2) in steady state. Like those spectra obtained for methionine and vinylglycine processing, the changes shown in Figure 1B are characterized by a loss of the 418-nm absorbance and by accumulation of a 490-nm band. There is a single isosbestic point at 460 nm. Note, however, that by contrast to Figure 1A the loss of the 418-nm species and the gain in 490-nm absorbance are not in synchrony with one another. Indeed, the 490-nm chro-

³ There are some kinetic differences which distinguish Figure 1A from the spectra obtained for vinylglycine turnover. The rate of formation of the 485-nm band is slower for 1 than for methionine, and the steady state does not persist for as long a period, even under conditions of identical enzyme-substrate concentrations and at the same temperature. The later, of course, reflects different V_{\max} values.

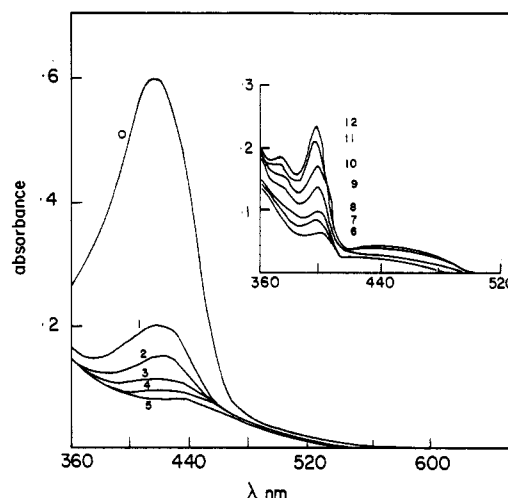


FIGURE 2: Absorbance spectra obtained during methionine γ -lyase catalyzed turnover of 2-amino-4-methoxy-3-*trans*-butenoate (3). Curve 0 was obtained from a 400- μ L solution containing 1.1 mg of enzyme, prior to addition of substrate. Curves 1–5 were obtained 1, 3, 7, 10, and 15 min after the addition of 100 μ L of 25 mM 3 (final concentration 5 mM), and curves 6–12 (inset) were obtained 20 and 30 min and 1, 2, 3, 7, and 20 h after addition of 3 (50 mM KPP_i, pH 8.3, 25 °C).

mophore appears only during “recovery” of the 418-nm band (curves 3–5) and, thus, appears to characterize the *emergence* from steady state. Ultimately, the 490-nm chromophore dissipates and a spectrum like that of curve 1 is obtained (data not shown).

Figure 2 gives the spectral data for processing of 2-amino-4-methoxy-3-*trans*-butenoate (3). There is a rapid loss of the 418-nm pyridoxalimine chromophore; within 15 min this absorbance has all but disappeared (curve 5). Then follows a very slow accumulation of a new spectral band which peaks at \sim 390 nm (inset). Spectra taken during the course of reaction (e.g., curves 11 and 12) display fine structure including an additional peak around 370 nm and a broad shoulder between 430 and 470 nm. We have followed these spectral changes for as long as 30 h and have not observed the return of the “resting” pyridoxalimine enzyme chromophore (curve 0). Curve 12 represents the peak 390-nm absorbance obtained during the course of reaction, during which time the enzyme retained full catalytic activity. Complete turnover of 3 (and, thus, emergence from steady state) would require \sim 7 h at 37 °C, given the V_{\max} value of Table I and the concentrations used in the experiment described by Figure 2. The duration of the steady state is likely to be considerably longer at 25 °C.

After incubation with 3 for 36 h, the enzyme solution was dialyzed overnight against 50 mM KPP_i buffer, 50 μ M PLP, and 1.0 mM 2-mercaptoethanol. The dialyzed enzyme was seen to be isospectral⁴ with curve 0 of Figure 2 and to have retained full catalytic activity.

The formation of α -ketovalerate from 2-amino-3-*trans*-pentenoate (4) is characterized by the development of a single absorbing species with λ_{\max} around 485–490 nm (Figure 3A). There is a concomitant loss of the 418-nm pyridoxalimine band. A single isosbestic point is seen at 460 nm. The complete turnover of 4 to product keto acid is coincident with the recovery of the 418-nm species (data not shown).

Ketovalerate formation from 2-amino-3-*cis*-pentenoate (5) shows the development of two high-wavelength absorbing

⁴ The total absorbance at 418 nm recovered after dialysis was corrected for dilution on the basis of protein absorbance at 280 nm.

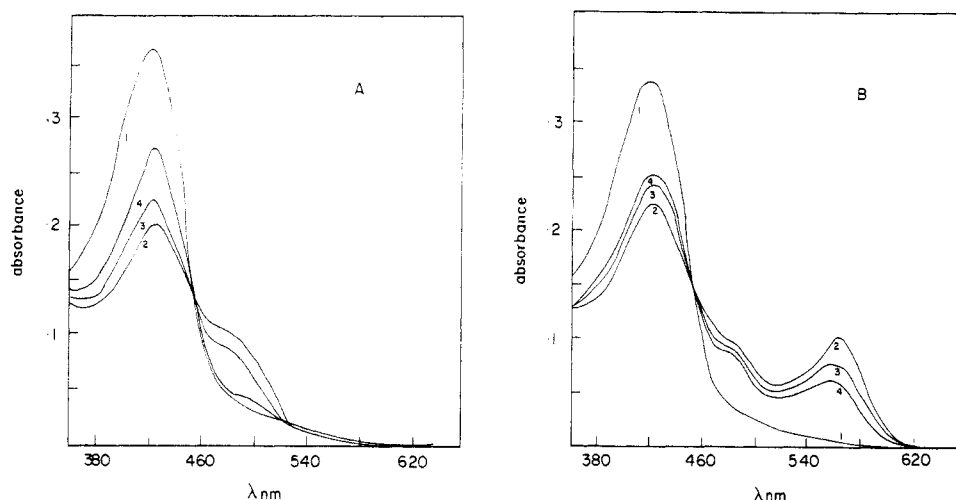


FIGURE 3: (A) Absorbance spectra obtained during formation of α -ketovaleate from 2-amino-3-*trans*-pentenoate (4). Curve 1 was obtained from a 400- μ L solution containing 675 μ g of methionine γ -lyase. Curves 2–4 were obtained 2, 5, and 15 min after the addition of 100 μ L of 250 mM 4 (final concentration 50 mM) (50 mM KPP_i, pH 8.3, 25 °C). (B) Absorbance spectra obtained during formation of α -ketovaleate from 2-amino-3-*cis*-pentenoate (5). Curve 1 was obtained from a 400- μ L solution containing 675 μ g of methionine γ -lyase. Curves 2–4 were obtained 2, 5, and 10 min after the addition of 100 μ L of 250 mM 5 (final concentration 50 mM) (50 mM KPP_i, pH 8.3, 25 °C).

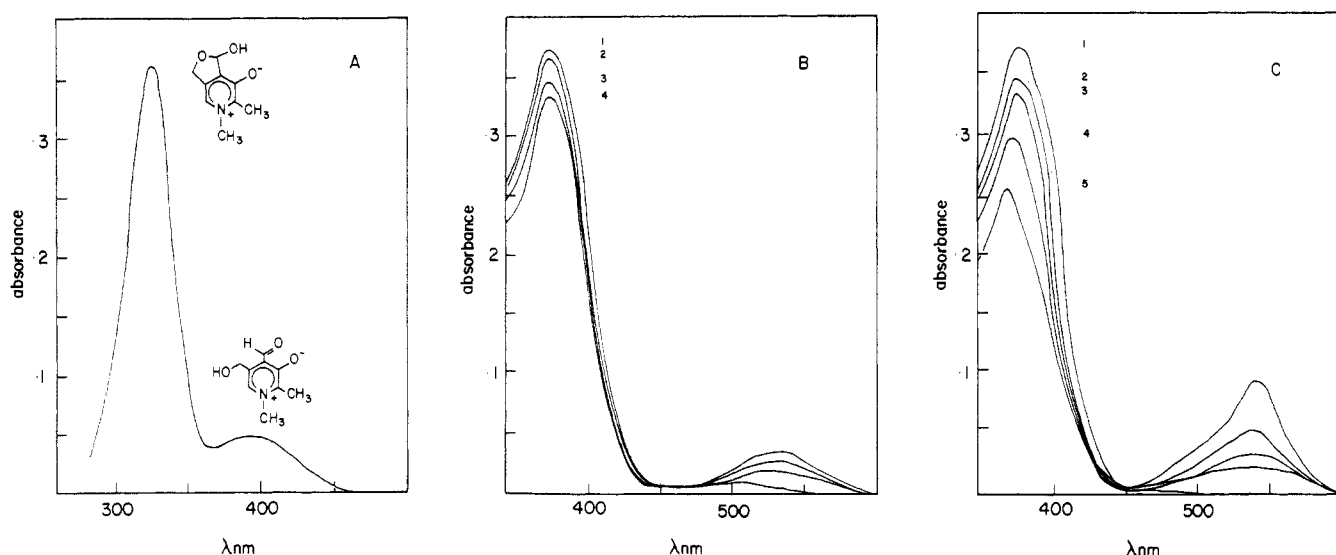


FIGURE 4: (A) Absorbance spectrum of pyridoxal methochloride (0.1 mM) in ethanol and 0.2 mM KOH. (B) Absorbance spectra obtained for reaction of 2-amino-3-*trans*-pentenoate (4) with pyridoxal methochloride. Curve 1 was obtained following the incubation of 100 mM 4 with 0.1 mM pyridoxal methochloride in alkaline (0.2 mM KOH) methanol for 1 h at 20 °C. Curves 2–4 were obtained 1, 5, and 10 min after addition of 0.1 mM Al(NO₃)₃. (C) Absorbance spectra obtained during reaction of 2-amino-3-*cis*-pentenoate (5) with pyridoxal methochloride. Curve 1 was obtained following the incubation of 100 mM 5 with 0.1 mM pyridoxal methochloride in alkaline (0.2 mM KOH) methanol for 1 h at 20 °C. Curves 2–5 were obtained 5, 10, 15, and 30 min after addition of 0.1 mM Al(NO₃)₃.

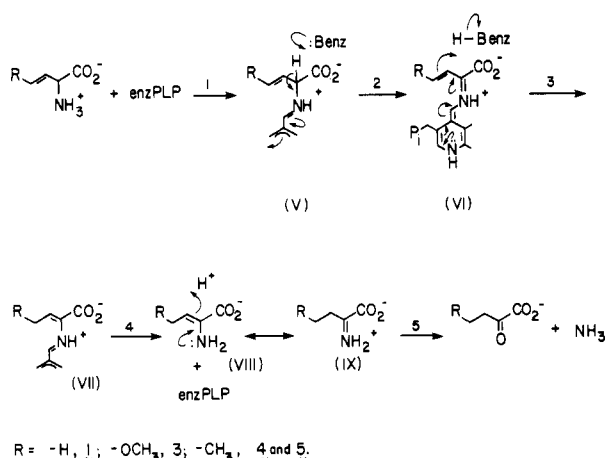
species (Figure 3B). There is a shoulder at ~ 490 nm and (for the first time) a second peak at 550 nm. There is an isosbestic point at 460 nm. We have followed the rate of accumulation of the 550-nm, chromophore together with the loss of catalytic activity during inactivation of the enzyme by 5. These rates are not synchronous. Under the reaction conditions described for Figure 3B, the 550-nm band gains its maximum absorbance within 10 min of addition of 5 to the enzyme, yet methionine γ -lyase lost only 10% of its native activity during this period. Since the extinction coefficient of the 550-nm band is unknown, we cannot evaluate the proportion of enzyme which exists as this species in the steady state. The 550-nm species persists until the enzyme is completely inactivated ($t_{1/2} = 110$ min at 50 mM 5), but fully inactivated enzyme lacks the 550-nm chromophore and shows only 25% of the native 418-nm aldimine absorbance.

Spectral Studies on the Model Reaction. Pyridoxal methochloride in alkaline methanol (0.2 mM KOH) has a major absorbance band in the far-ultraviolet, $\lambda_{\text{max}} = 328$ nm,

and a second visible band with $\lambda_{\text{max}} = 395$ nm (Figure 4A). The 328-nm species probably represents the pyridoxal methochloride hemiacetal, the predominant species at equilibrium. The 395-nm band is assumed to represent the free aldehyde, which should be the minor equilibrium component.

The addition of any of the amino acids of Table I to an alkaline methanol solution of pyridoxal methochloride results in the loss of the 328-nm species and an enhancement of absorbance at 395 nm, which is actually blue shifted to 388 nm. These spectral changes have been taken to characterize the formation of a Schiff's base adduct between the model pyridoxal and the α -amino nitrogen of substrate (Karube & Matsushima, 1976, 1977). In our experience, these spectral changes stabilize after ~ 1 h of incubation at 20 °C of the model pyridoxal and substrate amino acid. Thus, in the reactions described below, Al(NO₃)₃ catalyst was added only after the 388-nm band gave a stable absorbance yield. Initial spectral scans reported in the figure are those for solutions of pyridoxal methochloride and amino acids obtained only after

Scheme II



the 388-nm chromophore was fully developed.

Spectral data for the aluminum-catalyzed reactions of pyridoxal methochloride and the 2-amino-3-*trans*- and -*cis*-pentenoates (4 and 5, respectively) are given in Figure 4B,C. Both reactions are characterized by the loss of absorbance at 388 nm. For reaction with the *trans*-pentenoate (4), Figure 4B shows that there is a broad band of absorbance centered around 520–550 nm which develops simultaneously with loss of the 388-nm chromophore. This high-wavelength absorbance appears as a sharp peak at 550 nm for reaction of the model pyridoxal with the *cis*-pentenoate (5; Figure 4C). It is unclear why the two high-wavelength species generated should be different for the *cis* and *trans* compounds, but perhaps adduct geometry also influences electronic disposition, even in the model reaction. For both the *cis* and *trans* compounds, these chromophores persist for up to 24 h; the eventual loss of the high-wavelength species corresponds with partial recovery of the 388-nm peak and formation of a new absorbance at 348 nm (data not shown).

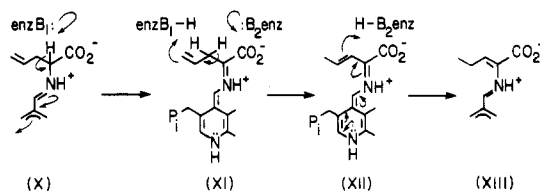
Analyses of reactions of pyridoxal methochloride with both L-methionine and vinylglycine (1) are characterized by two notable spectral events: (1) loss of the 388-nm pyridoxalimine methochloride chromophore and (2) simultaneous development of an absorbance band at 550 nm. The actual spectral tracings of these reactions are not shown here since our results, both for methionine and for vinylglycine, are identical with those previously reported by Karube & Matsushima (1977).

Discussion

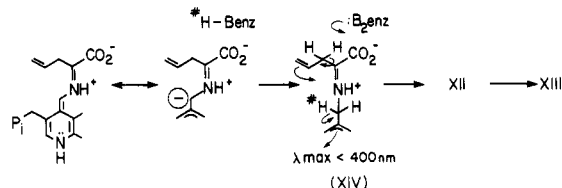
Enzymatic Reactions. (a) *Turnover.* Methionine γ -lyase will catalyze the net oxidative deamination of a variety of four- and five-carbon α -amino acids (1–5). The sequence outline in Scheme II accounts for the key mechanistic features of these reactions. The necessary events include (1) transaldimination between the enzyme-bound PLP and the α -amino nitrogen of the olefinic amino acid, (2) α -proton abstraction from the pyridoxalimine adduct V, (3) azaallylic isomerization, which converts the pyridoxalketimine paraquinoid VI to the enamine adduct VII, (4) reverse-transaldimination, giving the product enamine acid VIII and its tautomeric imino acid IX, and (5) hydrolysis to the keto acid and ammonia, which presumably occurs off the enzyme.

A number of enzymatic studies have suggested that intermediates such as VII will absorb in the region 485–500 nm (Guggenheim & Flavin, 1971; Davis & Metzler, 1972). We have similarly observed from the spectral data reported here that the reactions of methionine γ -lyase with both substrate methionine (Figure 1A) and with olefinic amino acids (Figures 1B and 3) are characterized by a loss of enzyme absorbance

Scheme III



Scheme IV



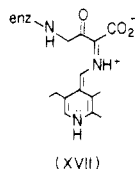
at 418 nm and accumulation of a transient absorber between 480 and 500 nm. Thus the enamine pyridoxalimine VII may be the consistently accumulating and kinetically competent steady-state intermediate formed during azaallylic isomerization. The extinction coefficient of the enamine adduct is unknown, so it is not possible to evaluate the contribution of VII to the population of possible steady-state intermediates. For methionine, vinylglycine (1), and the 2-amino-3-pentenoates (4 and 5), the conversion of VII to VIII may be at least partially rate determining (but, see below). The pyridoxal enamine adduct has been suggested to characterize the steady-state reactions of *Salmonella* cystathionine γ -synthetase with its substrate and several substrate analogues, including vinylglycine (Johnston et al., 1979a; Guggenheim & Flavin, 1971). The accumulating, steady-state species in these reactions similarly absorbs at 485 nm.

For the enzymatic reaction with allylglycine (2; the single γ,δ -olefinic acid examined) the dominating spectral event is loss of the 418-nm band; appearance of the 490-nm chromophore occurs only *during recovery* of the 418-nm species (Figure 1B). The high-wavelength species seems to signify the *emergence* from steady state, and, thus, the collapse of an enamine pyridoxalimine such as XIII cannot be solely rate determining. Indeed, Scheme III reminds one that the formation of the detected α -ketovalerate product, formed from allylglycine, is inherently more complicated mechanistically than those reactions outline by Scheme II. Both α - and β -proton abstraction (X \rightarrow XI \rightarrow XII) must precede azaallylic isomerization (XII \rightarrow XIII). This sequence requires further that reaction with allylglycine is distinct from those reactions with the β,γ -olefinic amino acids by requiring the accommodation of two substrate-derived protons (α and β) in the active site of the enzyme; that is, two enzymic bases are formally required for catalysis. If one of these is an enzyme amino acid side chain (enzB₁:) and the other (enzB₂:) is the benzylic carbon of the pyridoxal cofactor in its quinoid resonance contributor, then reaction with allylglycine might involve the intermediate formation of a pyridoxamine species (XIV)—a speculation outlined in Scheme IV. We do not wish to imply that Scheme IV is the only possible description of the pathway for allylglycine turnover. But it might be noted that XIV could be expected to give a pyridoxamine-like absorbance ($\lambda_{\max} < 400 \text{ nm}$), since protonation at the C₄' pyridoxal carbon interrupts the quinoid (XI) conjugation. Consonant with this interpretation are the spectral data of Figure 1B, which suggest that, in the steady state, most of the enzyme exists as a "silent" chromophore (i.e., $\lambda_{\max} < 400 \text{ nm}$). A sequence analogous to that of Scheme IV has been proposed previously to account for the spectral events which are observed for reaction of

O-succinyl-L-homoserine and cystathionine γ -synthetase in the γ elimination mode (Posner & Flavin, 1972; Johnston et al., 1979a). For net ketovalerate formation, the PNP oxidation state cannot persist. But conversion of XIV to XII may be a slow step, in accord with our spectral data (Figure 1B).⁵

The reaction of methionine γ -lyase with 2-amino-4-methoxy-3-*trans*-butenoate (**3**) also gives unusual spectra. There is a rapid loss of the 418-nm enzyme Schiff's base, but without accompanying formation of any high-wavelength species which otherwise would imply intermediate formation of one of the structures of Scheme II. Miles (1975) has reported the development of a 510-nm chromophore during reaction with **3** and tryptophan synthase and ascribes this peak to structure VI ($R = OCH_3$). Clearly, we detect no intermediate quinoid formation in our experiments. It is possible that the slowly accumulating 390-nm absorbance (Figure 2) is given by a noncovalent association of the enzyme and product VIII or IX. Since dialysis both dissipates the 390-nm band and restores the pyridoxalimine absorbance at 418 nm, the former would seem to be given by a small molecular weight species. It is possible that for reaction with **3**, each step in Scheme II is facile, except product release, which may be rate determining. We have not yet attempted to check this speculation kinetically.

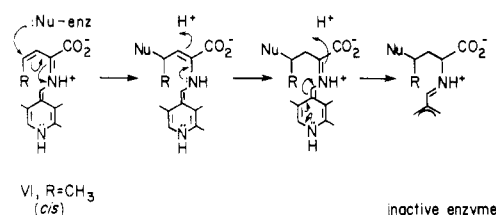
α -Proton abstraction from V (Scheme II) generates the β,γ -unsaturated pyridoxalketimine paraquinoid. For enzymes (e.g., cystathionine γ -synthetase) which catalyze both γ -elimination and γ -replacement reactions, VI ($R = H$) has been predicted to be the key partitioning intermediate, a strongly electrophilic species at C_4 upon which γ replacement takes place (Guggenheim & Flavin, 1971; Johnston et al., 1979a). The extended conjugation of this species is expected to push the ordinary quinoid absorbance (~ 500 nm) up to around 550 nm, but no 550-nm band has hitherto been observed for a γ -elimination enzyme (Davis & Metzler, 1972) during normal substrate turnover. Silverman & Abeles (1977) have shown the accumulation of a 550-nm band during inactivation of γ -cystathionase by trifluoroalanine. They suggest this absorbance is given by a covalent enzyme adduct (XVII), where



the pyridoxal paraquinoid equivalent is in conjugation with the γ -carbonyl of the derivatizing amide bond. The lack of any steady-state spectral evidence in favor of such a quinoid has been used to argue for the facile prototropic shift⁶ from VI to VII. We have recently suggested that this conversion is not only rapid but also effectively irreversible (Johnston et al., 1979a).

Note, however, from Figure 3B that reaction of methionine γ -lyase and 2-amino-3-*cis*-pentenoate (**5**) gives two high-wavelength absorbances, a shoulder at 485 nm and a sharp peak at 550 nm. We suggest that the 550-nm absorbance is given by the kinetically elusive β,γ -unsaturated paraquinoid VI ($R = CH_3$). Reaction of **4**, the *trans* equivalent of **5**, is, by contrast, altogether without a 550-nm band showing only

Scheme V

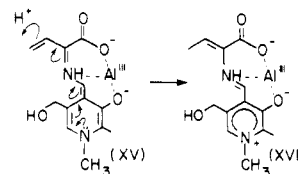


accumulation of the 485 nm-shoulder (probably given by VII). It would appear from these results that the different dispositions assumed in the active site by **4** and **5** serve to change the rate-determining step. That is, azaallylic isomerization (VI \rightarrow VII) is slow for the *cis* compound, but reverse transaldimination (VII \rightarrow VIII) is the rate-determining step for the *trans* molecule. Note from the data of Table II that α -proton abstraction (V \rightarrow VI) cannot be rate determining for either compound **4** or **5**.

(b) *Inactivation*. We suspect that these kinetic differences also account for the observation that the *cis*-olefin **5** is an irreversible inactivator of methionine γ -lyase but that the *trans*-olefin **4** is not. The quinoid VI ($R = CH_3$) is a very good Michael-type acceptor, and we imagine inactivation by nucleophilic addition to the C_4 methylene carbon (Scheme V).⁷ In principle the quinoids of both **4** and **5** are each mechanistically competent for interception by an adventitiously placed active-site nucleophile. But only when azaallylic isomerization is slow, that is, when 2-amino-3-*cis*-pentenoate is the substrate, is VI kinetically competent (i.e., it accumulates in the steady state) to serve as an inactivating electrophile. It is unclear why the *cis*-*trans* geometry should so alter the kinetic course of reaction, since the C_5 methyl group of either compound is in the same plane as the conjugated pyridoxal adduct (VI). But perhaps approach of the reprotonating base is sterically hindered when the substrate methyl group is in the *cis* orientation. One might also propose that the *trans* geometry hinders the approach of an alkylating nucleophile.

It is worth mentioning in passing that allylglycine neither inactivates methionine γ -lyase nor shows the development of a 550-nm chromophore during the course of reaction (Figure 1B). Therefore, on the basis of the preceding arguments, XII (Scheme IV) is the β,γ -unsaturated *trans*-quinoid, and it collapses to VII in the *trans* geometry.

Model Reactions. Karube & Matsushima (1977) have shown that pyridoxal methochloride will catalyze the nonenzymatic formation of α -ketobutyrate from vinylglycine (**1**). During the course of reaction, they observed the transient formation of a 550-nm absorbance to which they assigned structure XV, an adduct of the β,γ -unsaturated ketimine of



2-butenate and the aluminum chelate of pyridoxal methochloride paraquinoid. In the model reaction, one may assume that the kinetic constraints on reprotonation (XV \rightarrow XVI) arising from *cis*-*trans* isomerism are negligible. And we have reasoned, therefore, that the nonenzymatic reactions of **4** and

⁵ Azaallylic isomerization (XII \rightarrow XIII) must also be facile (as suggested for all compounds except the 2-amino-3-*cis*-pentenoate) since the only absorbing species of Figure 1B is a 490-nm band (XIII = VII).

⁶ Or equally rapid capture by adding a nucleophile in the γ -replacement mode.

⁷ It would seem, however, that the loss of catalytic activity should parallel the loss of absorbance at 550 nm if VI is the electrophile captured (vide supra).

5 should both proceed with formation of a chromophore around 550 nm, so long as reprotonation is slow. The results shown in Figure 4B,C bear out this prediction. It would appear, therefore, that stereochemical specificity (cis vs. trans isomerism) in the enzyme active site can radically alter the kinetic course of the reactions described. Thus, a substrate is converted into a potential inactivator by virtue of increased lifetime and so greater availability for capture.

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Spectroscopic Studies of Stellacyanin Derivatives[†]

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ABSTRACT: Two covalently modified derivatives of the apo-protein of the blue copper protein stellacyanin have been prepared. In one case, a dansyl group was linked to the cysteine at the copper binding site of apostellacyanin; in the other, a nitrophenol moiety has been attached to this same

cysteine. Fluorescence yields and emission maxima of the dansylated protein and pK determinations of the nitrophenol group linked to the protein suggest that the solvent microenvironment at the copper binding site of apostellacyanin is quite similar to bulk water.

The blue copper protein stellacyanin is found in the latex of the Japanese lacquer tree *Rhus vernicifera* (Reinhammar, 1970). Stellacyanin contains 40% carbohydrate by weight (Bergman et al., 1977) and one type-1 copper atom per molecular weight of 20 000 (Omura, 1961). The single polypeptide chain of 107 amino acid residues contains one cysteine (Cys-59), four histidines (His-32, -46, -92, and -100), and one cystine unit (linking Cys-87 and -93), but no methionine (Bergman et al., 1977). The absence of methionine distinguishes stellacyanin from two other blue copper proteins, plastocyanin (Colman et al., 1978) and azurin (Adman et al., 1978), whose X-ray crystal structures show two histidine

nitrogen atoms, one methionine sulfur atom, and one cysteine sulfur atom in the first coordination sphere of copper.

Crystallographic results are not available for stellacyanin, but physical studies have provided some information about the copper site in this metalloprotein. Cobalt substitution (McMillin et al., 1974), X-ray photoelectron spectroscopy (Wurzbach et al., 1977), and chemical modification studies with organomercurials (Morpurgo et al., 1972) have provided good evidence for cysteine sulfur coordination in stellacyanin, and ¹H NMR results (Hill & Lee, 1979) suggest the presence of two histidine ligands. Cystine disulfide ligation has been suggested (Ferris et al., 1978; Hill & Lee, 1979), but the evidence in this case is not as strong as for the cysteine ligand. Absorption and circular dichroism spectra of stellacyanin have been successfully interpreted in terms of the d-d and charge-transfer transitions expected for copper(II) in a flattened tetrahedral coordination environment containing both

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