A NEW TYPE OF PYRIDOXAL-P ENZYME CATALYZED REACTION: THE CONVERSION OF β,γ-UNSATURATED AMINO ACIDS TO SATURATED α-KETO ACIDS BY TRYPTOPHAN SYNTHASE

Edith Wilson Miles

Laboratory of Biochemical Pharmacology
National Institute of Arthritis, Metabolism, and Digestive Diseases
National Institutes of Health, Bethesda, Maryland 20014

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2-Amino-3-butenoic acid (vinyl glycine) and *trans*-L-2-amino-4-methoxy-3-butenoic acid are converted to saturated α-keto acids: α-ketobutyrate and α-keto-4-methoxybutyrate, respectively, by tryptophan synthase of *Escherichia coli*. A sequence of pyridoxal-P Schiff base intermediates is proposed for these reactions. The discovery of these pyridoxal-P dependent reactions of β,γ-unsaturated amino acids lends weight to previous proposals by others that pyridoxal-P Schiff base derivatives of β,γ-unsaturated amino acids are intermediates in γ-elimination reactions and in γ,β-isomerization reactions.

Pyridoxal-P enzymes catalyze a number of γ-elimination reactions (Reaction 1), γ-replacement reactions (Reaction 2), and γ,β-isomerization reactions (Reaction 3) (1-3). γ-Cystathionase from rats catalyzes Reaction 1 with cystathionine where X = $-SCH_2CHNH_2COOH$ or with homoserine where X = -OH (4). Cystathionine γ-synthase from *Salmonella* catalyzes Reaction 2 where X = $-OOCCH_2CH_2COOH$ and YH = cysteine (5). Threonine synthase from *Neurospora* catalyzes Reaction 3 where X = $-OPO_3H_2$ and Y = -OH (6).

$$XCH_2CH_2CHNH_2COOH + H_2O \longrightarrow XH + NH_3 + CH_3CH_2COCOOH \quad (1)$$

$$XCH_2CH_2CHNH_2COOH + YH \longrightarrow YCH_2CH_2CHNH_2COOH + XH \quad (2)$$

$$XCH_2CH_2CHNH_2COOH + YH \longrightarrow CH_3CHYCHNH_2COOH + XH \quad (3)$$

$$CH_2 = CHCHNH_2COOH + H_2O \longrightarrow CH_3CH_2COCOOH + NH_3 \quad (4)$$

Several authors have proposed that these reactions proceed through a series of pyridoxal-P Schiff base intermediates similar to those shown in Fig. 1 (1-3). The key common intermediate in all of these reactions is a pyridoxal-P derivative of vinyl glycine, Intermediate III. This paper shows that tryptophan

synthase of *E. coli* catalyzes Reaction 4 with 2-amino-3-butenoic acid (vinyl glycine) and an analogous reaction with *trans*-L-2-amino-4-methoxy-3-butenoic acid.[1] A probable mechanism for the reaction with vinyl glycine is also shown in Fig. 1. Reactions 1, 3, and 4 share 2 key intermediates, III and IV, and the pyridoxal-P catalyzed tautomerization of the double bond from the β,γ to the α,β position.

METHODS

2-Amino-3-butenoic acid (vinyl glycine) (7) and *trans*-L-2-amino-4-methoxy-3-butenoic acid (8, 9) were generous gifts of Dr. Robert R. Rando. Pyridoxal-P, α-ketobutyrate, α-hydroxybutyrate, and NADH were obtained from Sigma Chemical Company. [^{3}H]$NaBH_4$ (New England Nuclear), 250 Ci/mol, was used without dilution. The specific activity was estimated from the specific activity of [^{3}H]-pyridoxine-P which was prepared by the [^{3}H]$NaBH_4$ reduction of pyridoxal-P and was isolated by paper electrophoresis at pH 5.4 and elution. The concentration of the pyridoxine-P was determined from its absorbance spectra using the conditions and spectral data of Sober and Peterson (10). An aliquot of this solution was counted in 10 ml of Aquasol (New England Nuclear) in a Beckman LS-345 scintillation counter; the specific activity was 42,600 cpm/nmol. The β_2 subunit (11) and the $\alpha_2\beta_2$ complex (12) of tryptophan synthase were isolated as described previously. The concentration of α-ketobutyrate or the rate of its formation from vinyl glycine was determined from the decrease in absorbance at 340 nm of a reaction mixture containing 0.05 M Tris-HCl, pH 7.8; 0.01 M NH_4Cl; 0.05 mM pyridoxal-P; 0.1 mM NADH; excess lactic dehydrogenase (Worthington Biochemical Corp.); and vinyl glycine (0.01 M) where indicated. Phenylhydrazones were prepared by the method of Wada and Snell (13) for pyridoxal-P; the time of incubation was increased to 45 minutes. [^{3}H]-α-Hydroxy acids were prepared by treating 0.01 M standard α-keto acids in 0.1 ml of 0.1 M potassium phosphate, pH 7.8, or a reaction mixture in 0.1 ml 0.1 M potassium phosphate, pH 7.8, with 0.01 ml 0.1 M [^{3}H]$NaBH_4$, followed by addition of 0.01 ml concentrated HCl after 15 minutes. After standing in the hood, the reaction mixture was centrifuged and the supernatant solution was chromatographed on a 0.9 cm x 20 cm column of Sephadex G-25 fine in water. The radioactive fractions were combined, neutralized, and applied to a 1-ml column of Dowex 50-H^+ and washed with water. The radioactive fractions were concentrated to dryness, dissolved in 0.2 ml of methanol, and used for further identification. Paper electrophoresis of α-hydroxy acids on Whatman 3 MM paper was carried out for 1 hour at 2,500 V in pyridine : H_2O : glacial acetic acid (2.7 : 350 : 1) at pH 5.4 and 22°. Descending chromatography of α-hydroxy acids on Whatman 3 MM paper utilized 1-butanol : glacial acetic acid : H_2O (120 : 30 : 50). Radioactive areas on paper strips were located with a Vanguard Autoscanner 880. Phenylboronate derivatives of α-hydroxy acids were prepared and used for gas-liquid chromatography as described by Phillips (14).[2] Chemical ionization mass spectrometry using methane as the ionizing gas was used in conjunction with gas-liquid chromatography. A Finnegan Model 10150 gas chromatograph-mass spectrometer was utilized.[3] Direct

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[1] The measurement of NH_3 has not been carried out.

[2] I am grateful to Dr. Allen T. Phillips for suggesting this method and carrying out the analyses.

[3] These analyses were kindly performed by Mr. Noel F. Whittaker, Laboratory of Chemistry, National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health.

probe chemical ionization mass spectrometry using isobutane as the ionizing gas was performed on aliquots of methanolic solutions of α-hydroxy acids. Spectra of enzyme reaction mixtures (0.1 ml) were made using a microcell attachment in a Cary 14 spectrophotometer. Other spectral measurements were made in a Cary 11 or a Gilford instrument.

RESULTS

Reaction with trans-L-2-amino-4-methoxy-3-butenoic acid. Treatment of

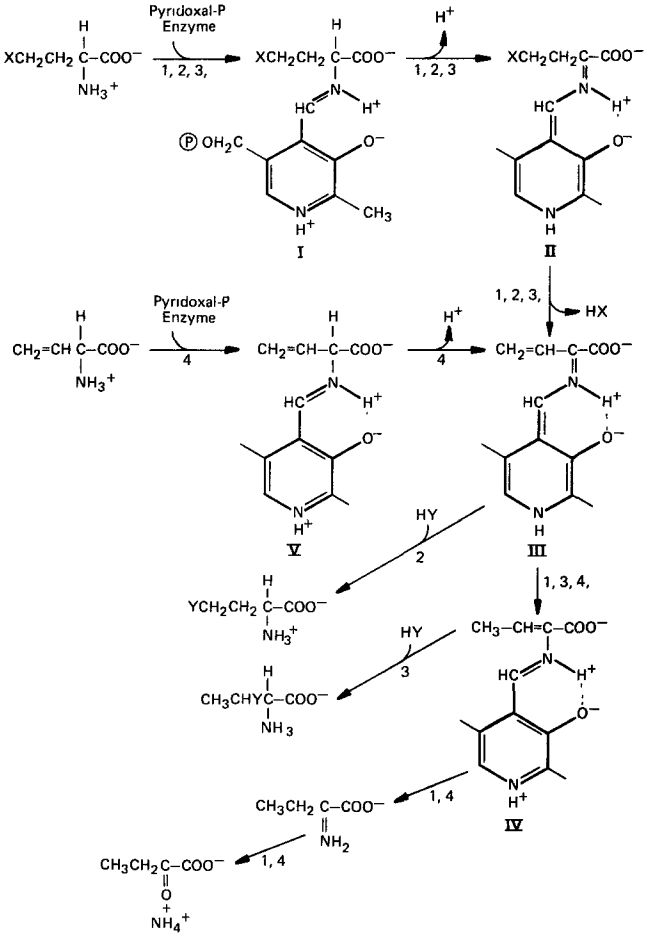

FIG. 1. *Postulated mechanisms for pyridoxal-P enzyme catalyzed reactions of γ-substituted amino acids and β,γ unsaturated amino acids (Reactions 1-4).* The first step in each reaction is the formation of an aldimine Schiff base (I or V) followed by loss of the α-hydrogen of the amino acid to form a quinoid structure (II or III). Elimination of the γ substituent X and a β proton from II yields the β,γ unsaturated imine III which can undergo γ-addition of Y to form $YCH_2CH_2CHNH_2COOH$ or tautomerization to yield the α,β unsaturated aldimine IV. IV can undergo β-addition to yield $CH_3CHYCHNH_2COOH$ or hydrolysis to yield α-ketobutyrate and NH_4^+. (Largely adapted from references 1 and 3.) The arabic numerals indicate steps in the corresponding reactions given in the text.

the $\alpha_2\beta_2$ complex of tryptophan synthase with *trans*-L-2-amino-4-methoxy-3-butenoic acid results in a series of spectral changes (Fig. 2) which suggest that this amino acid is a substrate, an inhibitor, or both. The presence of a product was detected by the spectrum of a phenylhydrazone derivative which is similar to the phenylhydrazone of pyruvate and α-ketobutyrate (see insert, Fig. 2). If the phenylhydrazone of the unknown compound is assumed to have the same extinction coefficient as pyruvate (1.8×10^4), then the concentration of product is about 0.005 M or about 50% of the starting material. Further conversion does not occur because the enzyme is irreversibly inactivated during the reaction.[4] The α-keto acid is not the product of a transamination reaction since stoichiometric amounts of α-keto acid and pyridoxamine-P are

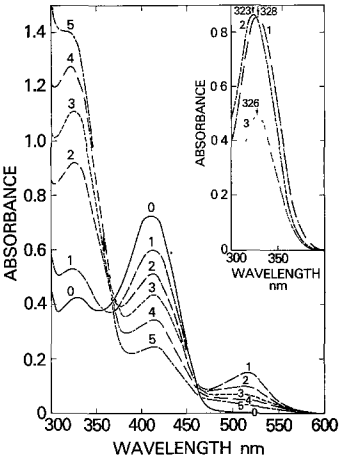

FIG. 2. *Treatment of the $\alpha_2\beta_2$ complex of tryptophan synthase with trans-L-2-amino-4-methoxy-3-butenoic acid.* Reaction mixtures contained in a total volume of 0.1 ml: $\alpha_2\beta_2$ complex (0.8 mg), potassium phosphate buffer, pH 7.8 (4 μmoles), and *trans*-L-2-amino-4-methoxy-3-butenoic acid (1 μmole). Spectra were recorded at 22° before addition of substrate (curve 0) and at the following times after addition of substrate: curve 1, 8 minutes; curve 2, 60 minutes; curve 3, 100 minutes; curve 4, 200 minutes; and curve 5, 280 minutes. After the last spectrum, an aliquot (0.005 ml) was diluted to 0.5 ml with 0.05 ml phenylhydrazine reagent and 0.445 ml H_2O. The spectrum was recorded after 45 minutes (insert, curve 1), as well as that of pyruvate (curve 2) and α-ketobutyrate (curve 3) treated similarly (0.5×10^{-4} M final concentration).

[4] A further investigation of this inactivation is in progress.

formed in transamination reactions and the concentration of the α-keto acid product is 80 times higher than the initial concentration of pyridoxal-P. A novel transamination reaction has previously been demonstrated to be catalyzed by the β_2 subunit of tryptophan synthase (15). The product was not a substrate for lactic dehydrogenase (see *Methods* and Table I). Treatment of the reaction

TABLE I: Identification of products of reaction of the $\alpha_2\beta_2$ complex with β,γ unsaturated amino acids

	Products from		Standards	
Methods	*trans*-L-2-amino-4-methoxy-3-butenoic acid	2-amino-3-butenoic acid	α-keto butyric acid	pyruvic acid
(1) Reaction with lactic dehydrogenase	-	+	+	+
(2) Phenylhydrazone and absorbance maximum (Fig. 2)	+ 327 nm	+ 325 nm	+ 325 nm	+ 323 nm
(3) [^{3}H]$NaBH_4$ reduction product:				
chromatography (BuA)	R_f = 0.78	R_f = 0.85	R_f = 0.85	R_f = 0.77
electrophoresis (pH 5.4)	24.5 cm	23 cm	23 cm	26 cm
GC-phenylboronate	-	6.45 min	6.45 min	4 min
GC-CI mass spectrum of phenylboronate	-	M + 1 = 191 (Fig. 3B)	M + 1 = 191	
CI mass spectrum (direct probe)	M + 1 = 135 (Fig. 3A)			

mixture with [^{3}H]$NaBH_4$ and isolation of the radioactive product (see *Methods*) gave 0.5 μmole of product (based on radioactivity) from 1.0 μmole of starting amino acid. The product gave a single peak on chromatography and electrophoresis (Table I). Since gas-liquid chromatography of the phenylboronate derivative gave no detectable peak[2,5], direct probe (isobutane) chemical ionization mass spectrometry was tried on the free acid. The results (Fig. 3A and Table I) show that the most abundant peak in the spectrum is at m/e 135 which is the expected M + 1 (M + H) for 2-hydroxy-4-methoxybutyrate.

Reactions with 2-amino-3-butenoic acid (vinyl glycine). The $\alpha_2\beta_2$ complex was treated with 2-amino-3-butenoic acid under the same conditions used in Fig. 2 except that 2 μmoles of DL-2-amino-3-butenoic acid replaced the substrate. The only spectral change observed was an increase in absorbance at about 320 nm of 0.13 which was complete in 30 minutes. No inactivation of the enzyme occurred. Preparation of the phenylhydrazone as described in Fig. 2 showed that a 37% conversion of DL-2-amino-3-butenoic acid to the presumed α-ketobutyrate product had occurred. The product was also a substrate for lactic dehydrogenase (see Table I and below). Treatment of the reaction mixture with [^{3}H]$NaBH_4$ and isolation of the radioactive product gave a yield of 0.5 μmole product (based on radioactivity) from 2.0 μmoles of racemic starting material. The product gave a single peak on chromatography and electrophoresis with a mobility identical to that of standard α-hydroxybutyrate. Gas-liquid chromatography of the phenylboronate derivative gave a single peak at 6.45 minutes, identical to that of the standard α-hydroxybutyrate phenylboronate. The gas chromatography--chemical ionization mass spectra (methane) of the phenylboronates of α-hydroxybutyrate and of the reduced reaction product were characterized by M + 1 (M + H) ion peaks at 190 and 191 (^{10}B and ^{11}B) in the expected ratio of 1 : 4 (Fig. 3B). The ion at 219 is the ethyl adduct ion, as expected using methane as reagent gas.

[5] The failure to detect a phenylboronate derivative could be due to a failure of the compound to form a derivative under these conditions or to a failure of the derivative to separate from the large reagent breakthrough peak.

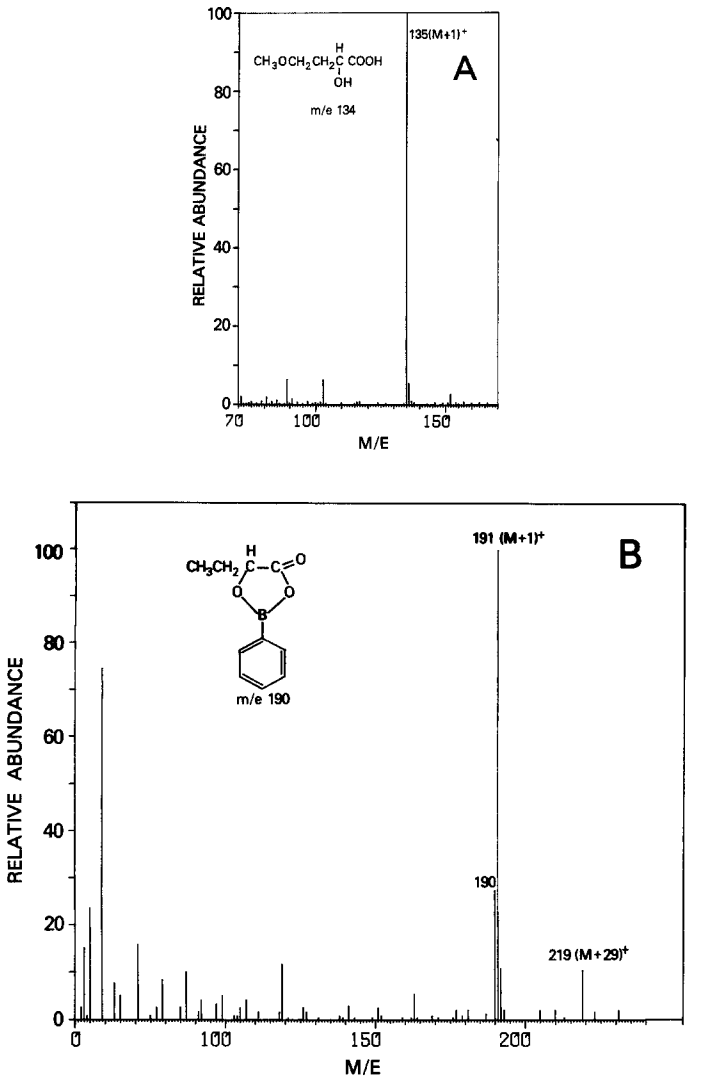

FIG. 3. *Chemical ionization mass spectra of the reduced product from trans-L-2-amino-4-methoxy-3-butenoic acid above m/e 70 (A) and of the phenylboronate of the reduced product from 2-amino-3-butenoic acid above m/e 50 (B).* Isobutane was the reactant gas in A; spectrum B was obtained in conjunction with gas chromatography using methane as the reactant gas. The spectrum of the phenylboronate of standard α-hydroxybutyrate was essentially identical to that in B.

The conversion of 2-amino-3-butenoic acid to α-ketobutyrate can also be measured in a coupled spectrophotometric assay using lactic dehydrogenase and NADH (see *Methods*). Using this assay it was shown that both the β_2 subunit and

the $\alpha_2\beta_2$ complex of tryptophan synthase convert 2-amino-3-butenoic acid to α-ketobutyrate. The rates are very slow (0.01 μmole/min/mg β_2 at 22° and 0.038 μmole/min/mg $\alpha_2\beta_2$ at 22°) but are proportional to enzyme concentration. The rate of conversion of serine to pyruvate by the β_2 subunit under the same conditions is about 30 times higher; the $\alpha_2\beta_2$ complex has no serine deaminase activity. In contrast, the conversion of *trans*-L-2-amino-4-methoxy-3-butenoic acid to 4-methoxy-α-ketobutyric acid is not carried out by the β_2 subunit alone.

DISCUSSION

The finding that β,γ-unsaturated amino acids are converted in pyridoxal-P dependent enzymatic reactions to α-keto acids is consistent with previous proposals that pyridoxal-P derivatives of β,γ-unsaturated amino acids are intermediates in γ-elimination reactions (1-3). This finding also extends our knowledge of the mechanism and reaction specificity of tryptophan synthase.

Tryptophan synthase has previously been shown to catalyze a wide variety of pyridoxal-P dependent reactions (16, 17). The $\alpha_2\beta_2$ complex carries out only β-addition reactions, whereas the β_2 subunit alone catalyzes β-addition reactions, β-elimination reactions, and a novel combined β-addition and transamination reaction (15). The discovery of these new reactions[6] extends the range of activities of tryptophan synthase previously known to include reactions in which the β,γ double bond is isomerized to an α,β double bond in the absence of any elimination or addition reactions. Although various enzyme-substrate intermediates of the β_2 subunit and $\alpha_2\beta_2$ complex have been observed or identified by their absorption peaks at 420, 325, and 468 nm (15, 17), the peak at 510 nm (Fig. 2) has not previously been observed. This peak is probably due to a highly conjugated pyridoxal-P derivative such as III or IV (Fig. 1).

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[6] After this manuscript was written I learned that these reactions are also carried out by sheep liver serine-threonine dehydratase, an enzyme which does not contain a pyridoxal-P cofactor (Kapke, G., and Davis, L., *Biochem. Biophys. Res. Commun.*, in press). I thank Dr. Davis for a preprint of this paper.

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