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Transamination Reaction Catalyzed by Kynureninase and Control of the Enzyme Activity†

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ABSTRACT: The inactivation of kynureninase occurred when the reaction was carried out in the absence of added pyridoxal 5′-phosphate. The degree of inactivation increased with the reaction time. The addition of either pyridoxal 5′-phosphate or pyruvate protected the enzyme from inactivation. Kynureninase was also inactivated by L-alanine, a reaction product, or by L-ornithine. The activity is restored by the addition of pyridoxal 5′-phosphate. Spectrophotometric studies on the inactivation indicate that the addition of L-alanine or L-ornithine to the holoenzyme leads to loss in the peaks at 337 and 430 mμ, and appearance of a new peak at 325 mμ. Apoenzyme was obtained by dialysis of L-ornithine-(or L-alanine)-treated enzyme. The apoenzyme is reactivated by pyridoxamine 5′-phosphate plus pyruvate, or by pyridoxal 5′-phosphate. Thus, the inactivation is due to formation of the bound pyridoxamine 5′-phosphate from the bound pyridoxal 5′-phosphate by transamination with L-alanine or L-ornithine. The product from L-ornithine was identified as Δ^1-pyrroline-2-carboxylic acid, the intramolecularly dehydrated form of α-keto-δ-aminovaleric acid. Kynureninase catalyzes an overall transamination between L-ornithine and pyruvate. There is close correlation between the amino acids that cause inactivation and those that transaminate, and between the α-keto acids that reactivate the inactivated enzyme and those that transaminate. The enzyme can act as an α-aminotransferase of high substrate specificity to regulate the enzyme activity by interconversion of the coenzyme moiety.

Previous articles have described the purification and crystallization of kynureninase from *Pseudomonas marginalis* and some of its properties (Moriguchi *et al.*, 1971a,b, 1973). The enzyme (mol wt 100,000) exhibits absorption maxima at 280, 337, and 430 mμ. One mole of pyridoxal-5′-P[1] is bound per mole of enzyme. The holoenzyme can be resolved to the apoenzyme by incubation with hydroxylamine, L-alanine, and L-ornithine, and reconstituted by the addition of pyridoxal-5′-P.

Recently, inactivation of kynureninase by L-alanine has been reported (Moriguchi *et al.*, 1971c). Kynureninase is inactivated by preincubation with a reaction product, L-alanine, while the addition of L-alanine to the assay system without preincubation causes only a slight decrease in the reaction rate. This activity is restored by the addition of pyridoxal-5′-P. Addition of L-alanine to the enzyme causes a decrease in absorbance at 430 mμ and appearance of a peak at 325 mμ. The inactivated enzyme is resistant to a borohydride reduction. The present investigation was undertaken to elucidate the mechanism of this inactivation and reactivation of the enzyme. Some studies on the regulation of kynureninase activity by the transamination between the enzyme-bound pyridoxal-5′-P (or pyridoxamine-5′-P) and an amino acid, *i.e.* L-alanine or L-ornithine (or pyruvic acid), are described here.

Experimental Section

Materials. D-Ornithine-HCl was prepared by hydrolysis of D-arginine-HCl with concentrated sodium hydroxide and

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[1] Abbreviations used are: pyridoxal-5′-P, pyridoxal 5′-phosphate; pyridoxamine-5′-P, pyridoxamine 5′-phosphate.

TABLE I: Inactivation of Enzyme by Preincubation with Various Amino Acids and Amines.[a]

Amino Acids or Amines	Rel Act. (%)
None	100
Glycine	100
L-Alanine	65
3-Guanidino-L-alanine	92
L-Serine	100
2-Aminoethylphosphonic acid	102
2-Amino-4-phosphonobutyric acid	102
β-Alanine	100
Taurine	100
Aminomethane sulfonic acid	100
3-Aminopropanesulfonic acid	100
L-α,β-Diaminopropionic acid	101
L-α-Amino-*n*-butyric acid	100
DL-β-Aminoisobutyric acid	97
L-α,γ-Diaminobutyric acid	98
γ-Aminobutyric acid	98
L-Ornithine	31
D-Ornithine	100
α-*N*-Acetyl-L-ornithine	100
δ-*N*-Acetyl-L-ornithine	87
L-Arginine	97
L-Citrulline	96
L-Canavanine	102
ε-Amino-*n*-caproic acid	97
L-Lysine	96
S-(β-Aminoethyl)-L-cysteine	100
L-Histidine	98
L-Phenylalanine	96
L-Aspartic acid	98
L-Threonine	97
L-Glutamic acid	97
Putrescine	97
Cadaverine	97

[a] The preincubation mixture contained 200 μmol of Tris-HCl buffer (pH 8.0). 1 μmol of the amino acid or amine, and 13 μg of enzyme in a volume of 3.05 ml. After preincubation at 25° for 1 hr, the reaction was started by the addition of L-kynurenine sulfate.

saturated barium hydroxide (Rivard, 1953). Authentic Δ^1-pyrroline-2-carboxylic acid and Δ^1-pyrroline-5-carboxylic acid were prepared enzymatically from D-proline and L-ornithine according to the procedure of Radhakrishnan and Meister (1958) and Soda and Misono (1968), respectively. Pyridoxamine-5′-P and sodium pyruvate were purchased from Sigma Chemical Co., St. Louis, Mo. α-Ketoglutaric acid and pyridoxal-5′-P were products of Kyowa Hakko Kogyo Co., Tokyo. 3-Aminopropanesulfonic acid, aminomethanesulfonic acid, and 3-guanidino-L-alanine were purchased from Aldrich Chemical Co., Madison, Wis., and 2-aminoethylphosphonic acid, 2-amino-4-phosphonobutyric acid, and L-canavanine from Calbiochem, Los Angeles, Calif. The other amino acids were products of Ajinomoto Co., Tokyo. L-[U-^{14}C]Ornithine was obtained from New England Nuclear, Boston, Mass. The other chemicals were analytical grade reagents.

Enzyme Preparation. Crystalline kynureninase was prepared from the cell-free extract of *Ps. marginalis* as described previously (Moriguchi *et al.*, 1971a,b).

TABLE II: Activation of Inactivated Enzyme by Pyridoxal-5′-P.[a]

Addition	Rel Act. (%)	
	− Pyridoxal-5′-P	+ Pyridoxal-5′-P
None	63	100
L-Ornithine	21	100
L-Alanine	40	100

[a] The preincubation mixture contained 200 μmol of Tris-HCl buffer (pH 8.0), 1 μmol of L-alanine or L-ornithine, and 13 μg of enzyme in a total volume of 2.85 ml. After preincubation at 25° for 1 hr, to the mixture was added 0.2 μmol of pyridoxal-5′-P. After incubation at 25° for another 10 min, the activity was determined.

Assay of Kynureninase. Kynureninase was assayed by measuring the rate of the decrease in absorbance at 360 mμ due to hydrolysis of kynurenine as reported previously (Moriguchi *et al.*, 1971a,b).

Protein Determination. Protein determination was performed by measuring the absorbance at 280 mμ ($E^{1\%}_{1\,cm}$ = 14.3).

Spectrophotometry. Spectrophotometric measurements were made with a Shimadzu MPS-50L recording spectrophotometer with a 1.0-cm light path.

Radioactivity Measurements. The radioactivity was measured with a Tri-Carb liquid scintillation 3320 spectrometer.

Results

Preincubation of Enzyme with Various Amino Acids. The activity of kynureninase was assayed after the enzyme was preincubated with various amino acids at pH 8.0 (Table I). L-Ornithine inactivated the enzyme more effectively than L-alanine. Preincubation with ε-*N*-acetyl-L-ornithine also led to partial inactivation of the enzyme, while D-ornithine, α-*N*-acetyl-L-ornithine, and the other amino acids had no effect. When the enzyme was preincubated with various concentrations of L-ornithine (0.5–5 μmol) at 30°, the activity of kynureninase decreased with increasing concentrations of L-ornithine and prolonged preincubation times (10–60 min) as previously demonstrated with L-alanine (Moriguchi *et al.*, 1971c). The addition of L-ornithine to the reaction mixture without preincubation brought about a slight decrease in the initial reaction rate. The enzyme activity was restored by subsequent addition of pyridoxal-5′-P (Table II). The spectrum of the enzyme was shifted by the addition of L-ornithine in the same way as observed when L-alanine was added (Moriguchi *et al.*, 1971c), but more rapidly: a decrease in absorbance at 430 mμ occurred with the concurrent appearance of a new peak at 325 mμ.

Dialysis of the enzyme incubated with L-ornithine or L-alanine against 0.01 M potassium phosphate buffer, pH 7.2, led to a complete loss of the characteristic absorption peak in the 325-mμ region and of the catalytic activity. The reduction of native enzyme with sodium borohydride led to the irreversible inactivation of enzyme. However, when the L-alanine-inactivated enzyme was treated with sodium borohydride, the activity was restored by the addition of pyri-

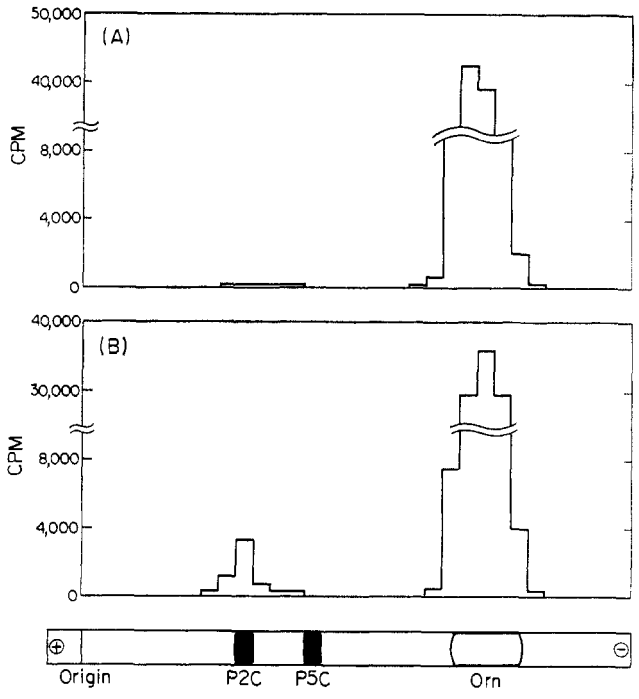

FIGURE 1: Paper electrophoresis of a product from L-ornithine. The supernatant of the reaction mixture was applied in 0.05-ml aliquots to a paper electrophoresis strip. Experimental details are given in the text. L-Ornithine, Δ^1-pyrroline-2-carboxylic acid, and Δ^1-pyrroline-5-carboxylic acid were also submitted to electrophoresis at the same time. After drying, the spots were visualized with ninhydrin and *o*-aminobenzaldehyde spray. Development with *o*-aminobenzaldehyde and ninhydrin showed orange and yellow colors, respectively, in the black areas. On development with ninhydrin, the light areas were violet, but were negative with *o*-aminobenzaldehyde. The paper strip (3 × 34 cm) was cut into serial 1-cm sections: P2C, Δ^1-pyrroline-2-carboxylic acid; P5C, Δ^1-pyrroline-5-carboxylic acid; (A) enzyme omitted, (B) complete system.

doxal-5′-P (Moriguchi *et al.*, 1971c). The enzyme treated with L-ornithine was also protected from the irreversible inactivation by sodium borohydride. These findings suggest that the inactivation of enzyme by incubation with L-ornithine or L-alanine involves the modification of the coenzyme moiety by the amino acids.

Protective Effect of Pyruvate on the Inactivation of Enzyme by Preincubation with L-Ornithine. When the enzyme was preincubated with L-ornithine in the presence of various concentrations of pyruvate, the prevention of inactivation of the enzyme was observed. The protective effect increased with increasing concentrations of pyruvate (Table III). Pyruvate had no influence on the initial rate of reaction with the native enzyme even at a considerably higher concentration, *e.g.*, 3.2×10^{-3} M. The results obtained suggest that the bound pyridoxal-5′-P may be converted by transamination with L-ornithine and L-alanine into the bound pyridoxamine-5′-P, and that the pyridoxal-5′-P may be regenerated by the reverse transamination between the pyridoxamine-5′-P and pyruvate. To confirm this mechanism attempts were made to isolate and identify the reaction products.

Identification of Transamination Products. PRODUCT FROM ORNITHINE. The product derived from L-ornithine by reaction with kynureninase was examined as follows. The reaction mixture consisting of 222 μg of enzyme, 20 μmol of Tris-HCl buffer (pH 8.0), 5 μmol of L-ornithine, 0.0049 μmol (0.001 mCi) of L-[U-^{14}C]ornithine, and 0.2 μmol of pyridoxal-5′-P

TABLE III: Effect of the Concentration of Pyruvate on the Protection of the Enzyme from Inactivation by Preincubation with L-Ornithine.[a]

Amount of Pyruvate (μmol)	Rel Act.[b] (%) L-Ornithine + Pyruvate	Rel Act.[b] (%) Pyruvate
0	30	
1	30	100
3	58	100
5	63	99
7	70	100
10	88	100

[a] The preincubation mixtures consisted of 200 μmol of Tris-HCl buffer (pH 8.0), 1 μmol of L-ornithine, the indicated amount of pyruvate, and 11 μg of enzyme in a total volume of 3.05 ml. After the preincubation at 25° for 1 hr, the reaction was initiated by the addition of L-kynurenine sulfate. [b] Relative activity is shown on the basis of activity obtained in the absence of L-ornithine and pyruvate (100).

in a total volume of 0.48 ml was incubated at 30° for 2.5 hr. A blank lacking the enzyme also was run. A yellow color of the reaction mixture, which is derived from pyridoxal-5′-P and its Schiff base, disappeared in the course of incubation. The reaction was stopped by the addition of 0.02 ml of 50% trichloroacetic acid. The supernatant solution obtained by centrifugation was examined by high voltage paper electrophoresis conducted in 1 N formic acid at 1000 V for 2 hr. The product from L-ornithine was allowed to react with *o*-aminobenzaldehyde and ninhydrin to yield an orange and a yellow color, respectively, as well as authentic Δ^1-pyrroline-2-carboxylic acid and Δ^1-pyrroline-5-carboxylic acid. The product migrated the same distance as authentic Δ^1-pyrroline-2-carboxylic acid and was separated clearly from Δ^1-pyrroline-5-carboxylic acid, as shown in Figure 1. The formation of that compound was not observed in the blank system. These findings suggest that Δ^1-pyrroline-2-carboxylic acid is produced in the reaction of L-ornithine with the enzyme.

PRODUCT FROM PYRIDOXAL-5′-P. As the spectral observations described above indicate that pyridoxamine-5′-P is probably formed in the reaction, the product from pyridoxal-5′-P was identified explicitly as follows. The reaction mixture from which the labeled ornithine was omitted was incubated as just mentioned. When electrophoresis of an aliquot of the deproteinized incubation mixture was carried out in 1 N formic acid, a new spot was observed, which is ninhydrin positive (an orange color) and migrated in the same manner as authentic pyridoxamine-5′-P. These results indicate the occurrence of an enzymatic α-transamination between the bound pyridoxal-5′-P and L-ornithine to form pyridoxamine-5′-P and α-keto-δ-aminovalerate, which is spontaneously converted into Δ^1-pyrroline-2-carboxylic acid.

PRODUCT FROM L-ALANINE. The reaction mixture containing 20 μmol of Tris-HCl buffer (pH 8.0), 10 μmol of L-alanine, 0.5 μmol of pyridoxal-5′-P, and 406 μg of enzyme in a total volume of 0.55 ml was incubated at 25° for 2 hr. After the reaction was terminated by the addition of 0.02 ml of 50% trichloroacetic acid, the supernatant was examined by paper chromatography with a solvent, *n*-butyl alcohol–water–ethanol (5:4:1) at about 20°. The developed and dried paper

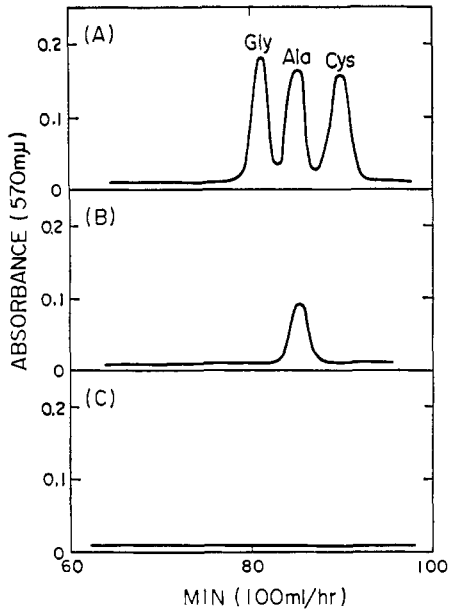

FIGURE 2: Ion-exchange chromatography of a product from pyruvate. The supernatant was examined by an automatic amino acid analyzer (Yanagimoto LC-5S) using an Aminex A-4 column (1.4 × 70 cm). The column was eluted with 0.2 M sodium citrate buffer, pH 3.25, at 50°. The flow rate was 100 ml/hr. Other conditions are given in the text: (A) authentic glycine (Gly), alanine (Ala), and cysteine (Cys); (B) complete system; (C) enzyme omitted.

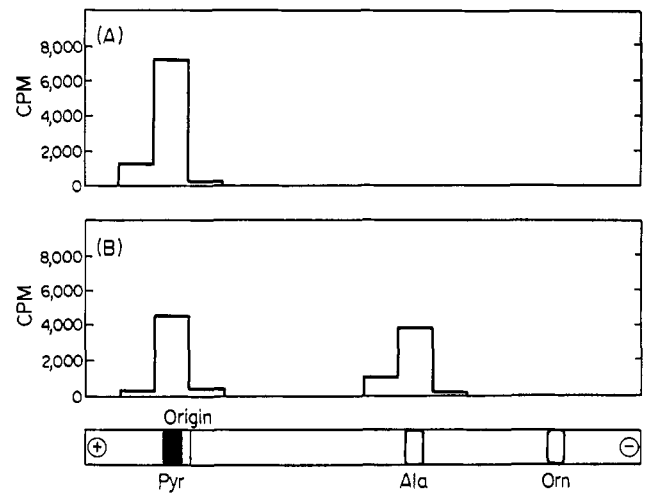

FIGURE 3: Incorporation of radioactivity into the product from [1-^{14}C]pyruvate. The reaction mixture contained 20 μmol of Tris-HCl buffer (pH 8.0), 10 μmol of L-ornithine, 10 μmol of pyruvate, 8 nmol (0.49 μCi) of [1-^{14}C]pyruvate, 0.04 μmol of pyridoxal-5′-P, and 1.386 mg of enzyme in a final volume of 0.39 ml. After incubation at 25° for 1 hr, the reaction was terminated by the addition of 0.01 ml of 50% trichloroacetic acid. Electrophoresis was conducted in 8% formic acid at 1 kV for 60 min: (A) enzyme omitted; (B) complete system.

was sprayed with a solution of 0.05% 2,4-dinitrophenylhydrazine in 2 N HCl, and dipped in 2% sodium hydroxide solution in 90% ethanol after drying. The product gave a yellow color with 2,4-dinitrophenylhydrazine and a red-brown color by further treatment with sodium hydroxide (2%). Thus, the product was paper chromatographically identified with authentic pyruvate (R_F 0.31).

PRODUCT FROM PYRUVATE IN OVERALL TRANSAMINATION. The reaction mixture contained 40 μmol of Tris-HCl buffer (pH 8.0), 10 μmol of L-ornithine, 10 μmol of pyruvate, 0.25 μmol of pyridoxamine-5′-P, and 1.84 mg of enzyme in a final volume of 1.02 ml. The reaction was incubated at 30° for 2 hr. After deproteinization by the addition of 0.04 ml of 50% trichloroacetic acid, followed by centrifugation, the supernatant was adjusted to about pH 3.52 and analyzed with an automatic amino acid analyzer (Yanagimoto LC-5S) (Figure 2). The amino acid formed was identified as alanine. The product was also identified as alanine by electrophoresis of the reaction system in which L-[^{14}C]pyruvate was incubated with L-ornithine (Figure 3). This finding confirms the conclusion that transamination between the α-amino group of L-ornithine and pyruvate is catalyzed by kynureninase.

Activation of Apoenzyme by Incubation with Pyridoxal-5′-P, Pyridoxamine-5′-P, and α-Keto Acids. An attempt was made to reconstitute the apoenzyme by vitamin B_6 derivatives and various α-keto acids (Table IV). The activity was restored by the addition of pyridoxal-5′-P, but not by the addition of pyridoxamine-5′-P or an α-keto acid alone. Preincubation of the apoenzyme with both pyridoxamine-5′-P and pyruvate also resulted in the reappearance of activity, but the combinations of pyridoxamine-5′-P and the other α-keto acids were not effective. These findings suggest that the pyridoxamine-5′-P form of enzyme is first formed from the apoenzyme by incubation with pyridoxamine-5′-P and then converted enzymatically into the pyridoxal-5′-P enzyme by transamination with pyruvate, which is the exclusive amino acceptor.

Additional evidence for enzymatic transamination between pyruvate and pyridoxamine-5′-P enzyme was obtained spectrophotometrically (Figure 4). The addition of pyruvate to the pyridoxamine-5′-P enzyme led to loss in absorption at 325 mμ, which is attributed to a bound pyridoxamine-5′-P, and appearance of a peak in the region of 430 mμ. No ap-

TABLE IV: Activation of Apokynureninase by Incubation with Pyridoxal-5′-P, Pyridoxamine-5′-P, and α-Keto Acids.[a]

Additions	Rel Act. (%)
Pyridoxal-5′-P	100
Pyruvate	0
Pyridoxamine-5′-P and pyruvate	86
α-Ketoglutarate	0
Pyridoxamine-5′-P and α-ketoglutarate	0
β-Phenylpyruvate	0
Pyridoxamine-5′-P and β-phenylpyruvate	0
Oxalacetate	0
Pyridoxamine-5′-P and oxalacetate	0
α-Ketobutyrate	0
Pyridoxamine-5′-P and α-ketobutyrate	0
Glyoxylate	0
Pyridoxamine-5′-P and glyoxylate	0
Pyridoxamine-5′-P	0
None	0

[a] The preincubation mixture contained 200 μmol of Tris-HCl buffer (pH 8.0), 5 μmol of an α-keto acid or 0.1 μmol of pyridoxamine-5′-P or 0.2 μmol of pyridoxal-5′-P (or both), and 12 μg of apoenzyme in a final volume of 3.05 ml. The preincubation was carried out at 25° for 1 hr. The reaction was started by the addition of L-kynurenine sulfate to determine the activity.

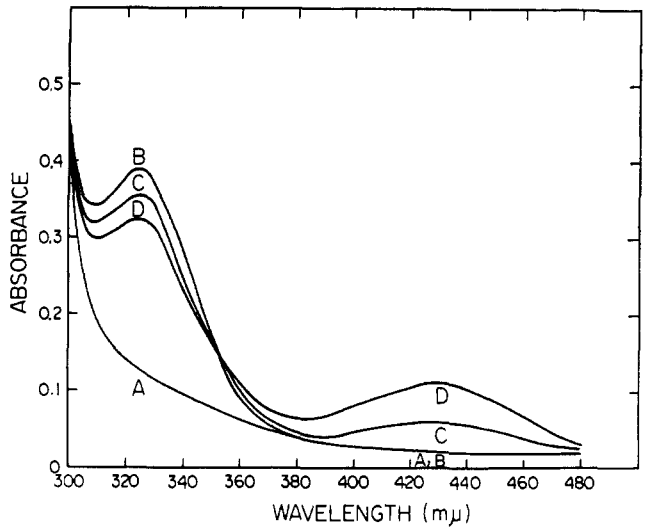

FIGURE 4: Spectral shifts of enzyme on addition of pyridoxamine-5′-P and pyruvate: curve A, a 0.096% solution of apoenzyme in 0.03 M Tris-HCl buffer, pH 8.0; curve B, enzyme solution used in curve A after addition of 3×10^{-5} M pyridoxamine-5′-P and incubation at 25° for 20 min; curve C, enzyme solution used in curve B, 1 hr after addition of pyruvate (final concentration, 3×10^{-3} M); curve D, enzyme solution used in curve B, 4.5 hr after addition of pyruvate. The spectra were corrected for the resulting dilution.

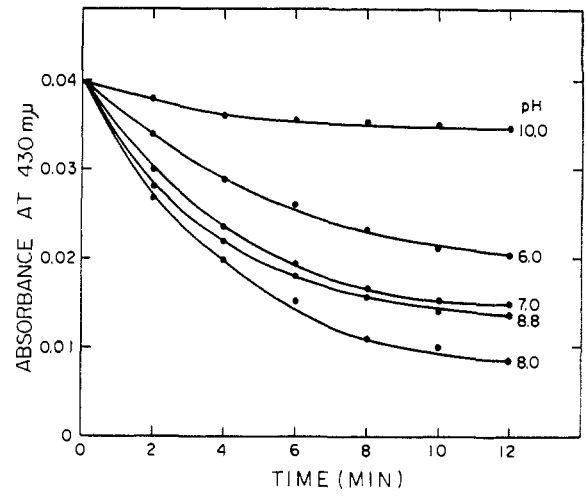

FIGURE 5: Effect of pH on the rate of transamination. The reaction mixture contained 227 μg of enzyme, 20 μmol of L-alanine, and 20 μmol of the following buffers in a volume of 0.4 ml: potassium phosphate buffer, pH 6.0 and 7.0; Tris-HCl buffer, pH 8.0 and 8.8; borate buffer, pH 10.0. The rate of transamination was determined by measuring a decrease in absorbance at 430 mμ.

preciable spectral change was observed by the addition of the other α-keto acids.

Effect of pH on the Rate of Transamination. The optimum pH of conversion of the pyridoxal-5′-P form of enzyme into the pyridoxamine-5′-P form of enzyme by L-alanine was examined by spectrophotometry. As shown in Figure 5, the maximum rate was observed at pH 8.0, which is the optimum pH for the kynureninase reaction (Moriguchi *et al.*, 1971 a,b).

Effect of L-Ornithine, Pyruvate, and Pyridoxal-5′-P on Kynureninase Reaction. The time course of kynureninase reaction in the presence of L-ornithine, pyruvate, or pyridoxal-5′-P (or all) is given in Figure 6. The initial rates with no addition (curve 1) and that with added pyridoxal-5′-P alone (curve 2) were similar. In the experiment with no addition, the rate of kynureninase reaction was initially linear with time but then declined. Addition of pyridoxal-5′-P and pyridoxal-5′-P plus pyruvate stimulated effectively the kynureninase reaction to the same extent (curves 2 and 6) and the reaction proceeded linearly for more than 15 min under the conditions. The presence of pyruvate prevented the reaction from declining with time (curve 4). When the enzyme was incubated in the presence of L-ornithine, the reaction fell off markedly (curve 3). Subsequent addition of pyridoxal-5′-P resulted in a substantial increase in the reaction rate (curve 3′), which was almost the same as that obtained when pyridoxal-5′-P was presented initially (curve 2). The presence of pyruvate in the reaction system protected the enzyme from inactivation by L-ornithine (curve 5). These findings are compatible with the results described above.

Discussion

The kynureninase reaction in the absence of added pyridoxal-5′-P was initially linear with time for a short time but then fell off. However, when pyridoxal-5′-P was added to the reaction mixture, the reaction proceeded linearly. The addition of L-alanine or L-ornithine to the reaction mixture led to a reversible inactivation of the enzyme and the degree of inactivation increased with increasing concentration of the amino acids. Pyruvate protected the enzyme from such inactivation. Loss of kynureninase activity by L-ornithine or L-alanine was associated with formation of a new absorption peak with a maximum at about 325 mμ, and disappearance of the peak at 430 mμ. The former absorption peak is characteristic of both free and bound pyridoxamine-5′-P (Peterson and Sober, 1954). Subsequent dialysis of the inactivated enzyme resulted in loss of the absorption at 325 mμ to give the apoenzyme. The addition of both pyruvate and pyridoxamine-5′-P to the apoenzyme caused a decrease in absorbance at 325 mμ and an increase at 430 mμ with concomitant reconstitution of the activity, but neither pyridoxamine-5′-P nor pyruvate (or some other α-keto acids) alone had such an effect. These findings indicate that kynureninase catalyzes transamination between the enzyme-bound pyridoxal-5′-P and L-alanine (or L-ornithine) and also the reverse reaction between the enzyme-bound pyridoxamine-5′-P and pyruvate. These reactions can be regarded as half-reactions of the L-ornithine–pyruvate aminotransferase reaction. The overall reaction was also demonstrated. In the overall transamination between L-ornithine and pyruvate, the α-amino group of L-ornithine is enzymatically transferred to pyruvate to yield L-alanine and α-keto-δ-aminovalerate, which is spontaneously converted into the intramolecularly dehydrated form, Δ^1-pyrroline-2-carboxylate. Since the cyclic form is markedly favored under the conditions reported by Cabello *et al.* (1964), transamination between L-ornithine and pyruvate must be practically irreversible; thus, Δ^1-pyrroline-2-carboxylate probably does not participate in the regulation described below. The occurrence of α-transamination seems to be consistent with the observation that δ-*N*-acetyl-L-ornithine caused the inactivation (Table I) and the spectral shift of the enzyme, whereas α-*N*-acetyl-L-ornithine was not effective. Thus, it is suggested that kynureninase activity can be under the control of α-transamination catalyzed by the enzyme itself, although no *in vivo* evidence has been obtained for such regulation. These interconversion reactions can be summarized in Figure 7.

The resistance of the enzyme treated with L-alanine or L-

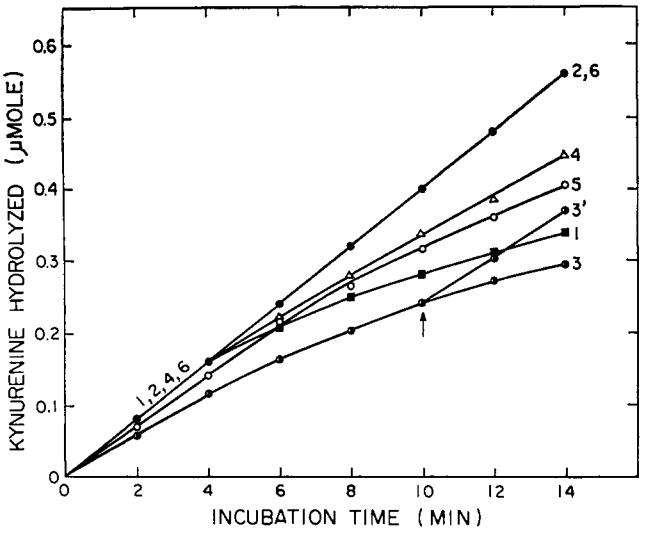

FIGURE 6: Effect of L-ornithine, pyruvate, and pyridoxal 5'-P on the kynureninase reaction. The reaction mixture contained 200 μmol of Tris-HCl buffer (pH 8.0), 1 μmol of L-kynurenine sulfate, and 0.2 μmol of pyridoxal-5'-P, 2 μmol of L-ornithine, or 5 μmol of pyruvate, and enzyme in a final volume of 3.1 ml. The incubation was carried out at 25°: curve 1, kynureninase reaction in the absence of added pyridoxal-5'-P, L-ornithine, and pyruvate; curve 2, the reaction in the presence of pyridoxal-5'-P; curve 3 the reaction in the presence of L-ornithine; curve 4, the reaction in the presence of pyruvate; curve 5, the reaction in the presence of L-ornithine and pyruvate; curve 6, the reaction in the presence of pyridoxal-5'-P and pyruvate. At the point indicated by the arrow, pyridoxal-5'-P was added to the enzyme of curve 3 (curve 3').

ornithine to the reduction with sodium borohydride is also explicable according to this mechanism; the pyridoxamine-5'-P moiety produced from the bound pyridoxal-5'-P by transamination is naturally not susceptible to the reduction.

Transamination of the coenzyme moiety of a pyridoxal-5'-P enzyme was first demonstrated in studies on bacterial aspartate β-decarboxylase by Meister and his coworkers (Miles *et al.*, 1968; Tate and Meister, 1971) who reported that the β-decarboxylation of aspartate is regulated by transamination between L-aspartate (or other L-amino acids) and pyruvate (or other α-keto acids); transamination of the enzyme with L-aspartate (or other L-amino acids) converts the enzyme-bound pyridoxal-5'-P into the enzyme-bound pyridoxamine-5'-P (Novogrodsky and Meister, 1964; Meister *et al.*, 1951 a,b; Novogrodsky *et al.*, 1963; Soda *et al.*, 1964; Tate and Meister, 1969, 1970). The enzyme acts both as an aspartate β-decarboxylase (and also cysteinesulfinate desulfinase) and as an aminotransferase of low substrate specificity, but of high optical specificity. Pyruvate is formed from hydrolysis of the enzyme-bound alanine–ketimine intermediate of the decarboxylation of aspartate. Thus, they proposed a novel type of allosteric regulatory mechanism in which the α-keto acid effector is produced from the substrate by the action of enzyme. A substantially analogous mechanism has been presented by Yorifuji *et al.* (1971) for regulation of arginine racemase of *Ps. graveolens*. Arginine racemase catalyzes α-transamination between D- or L-ornithine (or certain other L-amino acids, *e.g.*, 2,4-diaminobutyrate) and the bound pyridoxal-5'-P, and the reverse reaction between pyruvate (or certain other α-keto acids) and the bound pyridoxamine-5'-P to regulate the enzyme activity. Thus, the transamination of the coenzyme moiety catalyzed by kynureninase provides a third example for the homologous regulation mechanism of

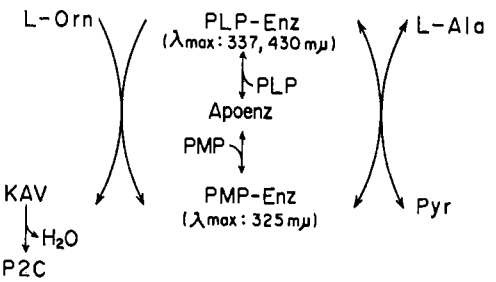

FIGURE 7: Scheme for interconversion of coenzyme of kynureninase: PLP, pyridoxal-5'-P; PMP, pyridoxamine-5'-P; KAV, α-keto-δ-aminovaleric acid; P2C, Δ^1-pyrroline-2-carboxylic acid; Ala, alanine; Orn, ornithine; Enz, kynureninase.

pyridoxal-5'-P enzymes, though certain differences are observed in the properties of transaminations by the enzymes.

Studies on the effects of pyridoxal-5'-P and pyruvate on the initial rate and the time course of kynureninase reaction in the presence of L-ornithine (Figure 6) revealed that the pyridoxamine-5'-P formed from the bound pyridoxal-5'-P by transamination may dissociate readily from the enzyme. This is consistent with the fact that the apoenzyme is obtainable by dialysis of the pyridoxamine-5'-P form of enzyme even in the presence of low concentrations of buffer (*e.g.*, 0.01 M) for 7–10 hr.

Inactivation of arginine racemase by incubation with L-ornithine is hardly protected by the presence of pyridoxal-5'-P even with high concentrations of the buffer, and exhaustive dialysis for a long period is required to resolve the pyridoxamine-5'-P form of enzyme to the apoenzyme (Yorifuji *et al.*, 1971). In the case of aspartate β-decarboxylase, pyridoxamine-5'-P dissociates from the enzyme only in the presence of high concentrations of buffer (Novogrodsky and Meister, 1964).

From the results described above, it is suggested that the strength of binding of pyridoxamine-5'-P to the apoenzyme may be of the order of that in arginine racemase, aspartate β-decarboxylase, and kynureninase. A product of kynureninase reaction, L-alanine, participates in the regulation system as a negative effector, and a positive effector, pyruvate, is produced from the L-alanine by the enzymatic transamination. The regulatory mechanism for kynureninase is unique in this respect. It seems of interest that ornithine serves as a potent negative effector for both arginine racemase and kynureninase, though either the L or D isomer is effective for the former and the L enantiomorph is exclusive for the latter enzyme. The physiological role of ornithine in the metabolic regulation of amino acids is not probably negligible from this point of view. Detailed investigation on the reaction mechanism of kynureninase, which was studied previously by a few groups (Jakoby and Bonner, 1953a,b; Braunstein and Shemyakin, 1953; Longenecker and Snell, 1955), is now in progress.

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Mechanism of Feedback Inhibition by Leucine. Binding of Leucine to Wild-Type and Feedback-Resistant α-Isopropylmalate Synthases and Its Structural Consequences†

Elizabeth Teng-Leary and Gunter B. Kohlhaw*

ABSTRACT: Binding of the feedback inhibitor, leucine, to a wild-type and a feedback-resistant form of α-isopropylmalate synthase from *Salmonella typhimurium* has been studied by equilibrium dialysis and ultraviolet difference spectroscopy. Binding of leucine to wild-type enzyme is positively cooperative, and the affinity for leucine increases with decreasing enzyme concentration. At a protein concentration of 1 mg/ml, the apparent binding constants for leucine are 10.5 μM for wild-type enzyme and about 1.5 mM for the feedback-resistant enzyme. This difference is similar to that observed in leucine inhibition kinetics. No more than two leucine sites per tetramer can be saturated. Binding of leucine has also been examined in the presence of either substrate. Acetyl-CoA has an antagonistic effect on leucine binding, yielding a biphasic Hill plot with mixed positive and negative cooperativity. α-Ketoisovalerate, the other substrate, increases the affinity for leucine, thereby lowering the positive cooperativity of leucine binding. A mixed effect is observed when leucine binding is studied in the presence of acetyl-CoA and α-ketoisocaproate, a very slowly reacting analog of the natural substrate α-ketoisovalerate. Ultraviolet absorption difference spectroscopy suggests that similar conformational changes occur upon binding of leucine to either the wild-type or the feedback-resistant enzyme. The concentrations of leucine required for a half-maximal effect in difference spectroscopy correlate closely with the apparent binding constants obtained in equilibrium dialysis experiments. The difference spectra observed upon binding of α-ketoisovalerate or acetyl-CoA are similar to each other and dissimilar to that caused by leucine. In contrast to wild-type α-isopropylmalate synthase, the feedback-resistant enzyme is not dissociated by inhibitory concentrations of leucine. The feedback resistance of the mutated enzyme is discussed in terms of strongly impaired leucine binding which is accompanied by the loss of preferential binding of the inhibitor to enzyme dimers.

For the past several years, this laboratory has been studying α-IPM[1] synthase, the first enzyme specific to leucine biosynthesis. The enzyme from *Salmonella typhimurium* is of particular interest because it is subject to an association–dissociation equilibrium which is affected by various ligands (Leary and Kohlhaw, 1970, 1972). Physical and chemical studies have indicated that the highest aggregate assumed by the native enzyme is a tetramer composed of very similar, if not identical polypeptide chains with a molecular weight of about 50,000 (Kohlhaw and Boatman, 1971; Bartholomew and Calvo, 1971; Leary and Kohlhaw, 1972). Kinetic studies have shown little or no cooperativity between substrate sites, but pronounced positive cooperativity between leucine sites, with

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[1] Abbreviation used is: α-IPM, α-isopropylmalate.