L-Lysine–α-Ketoglutarate Aminotransferase. II. Purification, Crystallization, and Properties*

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ABSTRACT: The preparation of crystalline L-lysine–α-ketoglutarate aminotransferase from *Achromobacter liquidum* is described. The enzyme is homogeneous by the criteria of ultracentrifugation, free-boundary electrophoresis, and disc gel electrophoresis. The molecular weight is 116,000, and 2 moles of pyridoxal 5′-phosphate are bound per mole of holoenzyme. The enzyme exhibits absorption maxima at 415 and 340 mμ. No appreciable spectral change was observed on varying pH. Addition of an amino donor to the enzyme produced a decrease in the absorbance at 415 mμ and an increase in that in the 340-mμ region with concomitant shift of the maximum from 340 to 330 mμ. The spectrum of the enzyme was not influenced by addition of α-ketoglutarate. Incubation of the enzyme with L-lysine in the presence of a high concentration of phosphate gave an inactive form of the enzyme (semiapoenzyme) which could be reactivated with pyridoxal 5′-phosphate. The absorption spectrum of semiapoenzyme does not have an absorption maximum at 415 mμ, but does have one at 340 mμ. This inactive form of enzyme contains 1 mole of pyridoxal 5′-phosphate. L-Lysine–α-ketoglutarate aminotransferase exhibits optimal activity at pH 8.3–8.5; it is stable over the pH range 6.0–7.5. It catalyzes transfer of the terminal amino groups of L-lysine and L-ornithine to α-ketoglutarate which is the exclusive amino acceptor. The following Michaelis constants were determined: L-lysine (2.8×10^{-3} M), α-ketoglutarate (5.0×10^{-4} M), and pyridoxal 5′-phosphate (3.57×10^{-7} M).

Although considerable effort has been devoted to the purification and the characterization of α-amino acid aminotransferases in recent years (Guirard and Snell, 1964; Meister, 1965; Fasella, 1967), little attention has been paid to the aminotransferase catalyzing the ε transamination of L-lysine. In previous work, evidence was obtained for the occurrence of enzymatic transamination between L-lysine and α-ketoglutarate in *Flavobacterium fuscum*, *Flavobacterium flavescens*, and *Achromobacter liquidum* (Soda *et al.*, 1961). Identification and characterization of the product derived from L-lysine have been undertaken in an effort to obtain information concerning which amino group of the amino donor is transaminated (Soda *et al.*, 1966, 1968). These investigations have revealed that the terminal amino group of L-lysine is enzymatically transferred to α-ketoglutarate to yield α-aminoadipate-δ-semialdehyde which is immediately converted into the intramolecularly dehydrated form, Δ^1-piperideine-6-carboxylic acid. In the present paper we report the preparation of crystalline L-lysine–α-ketoglutarate aminotransferase from *A. liquidum*, and some of its properties.

Experimental Section

Materials. *o*-Aminobenzaldehyde (Smith and Opie, 1955), ε-*N*-acetyl-L-lysine, α-*N*-acetyl-L-lysine (Neuberger and Sanger, 1943), and hydroxylapatite (Tiselius *et al.*, 1956) were prepared according to the methods given in the literatures. ε-*N*-Methyl-L-lysine-HCl was kindly supplied by Dr. N. L. Benoiton, University of Ottawa, Canada. DEAE-cellulose, sodium pyruvate, sodium α-ketobutyrate, oxaloacetic acid, α-ketocaproic acid, sodium α-ketovalerate, δ-aminovaleric acid, L-α,γ-diaminobutyric acid, pyridoxamine 5′-phosphate, pyridoxal-HCl, and pyridoxamine-2-HCl were purchased from Sigma Chemical Co., St. Louis, Mo. L-Lysine-HCl and L-arginine-HCl were obtained from Tanabe Seiyaku Co., Osaka. α-Ketoglutaric acid, L-valine, L-phenylalanine, ε-aminocaproic acid, and γ-aminobutyric acid were products of Ajinomoto Co., Tokyo. L-Aspartic acid and L-leucine were purchased from Kyowa Hakko Kogyo Co., Tokyo. L-α-Aminoadipic acid was a product of California Corp. for Biochemical Research, Los Angeles, Calif. Pyridoxal 5′-phosphate was a product of Dainippon Seiyaku Co., Osaka. Pyridoxal 5′-phosphate and pyridoxamine 5′-phosphate were chromatographically purified according to the method of Peterson and Sober (1954). The other chemicals were analytical grade reagents.

Methods. *A. liquidum* IFO 3084 was grown in the medium described previously (Soda *et al.*, 1968). The cultures were carried out in a 10-l. jar fermentor at 30° for 18 hr under aeration. The cells harvested were washed with 0.85% sodium chloride. The aminotransferase was assayed by measuring the amount of glutamate formed (procedure A) or by determining the amount of Δ^1-piperideine-6-carboxylic acid formed (procedure B) according to the methods given previously (Soda *et al.*, 1968). One unit of L-lysine–α-ketoglutarate aminotransferase is defined as the amount of enzyme that catalyzes the formation of 1.0 μmole of glutamate

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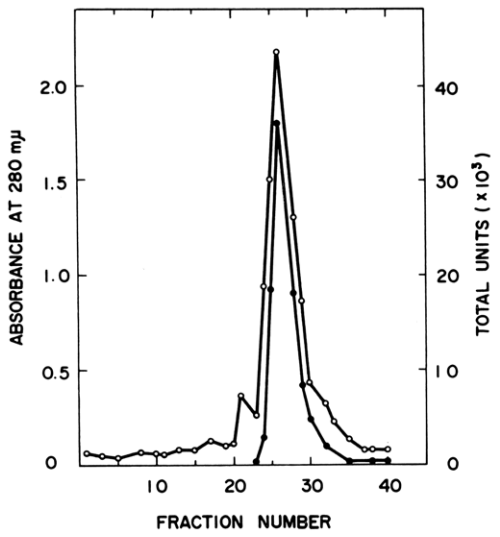

FIGURE 1: The elution pattern from a hydroxylapatite column. The flow rate was approximately 10 ml/hr and 10-ml fractions were collected. (○) Absorbance at 280 mμ; (●) L-lysine–α-ketoglutarate aminotransferase activity determined by procedure B. Other conditions are given in text.

or Δ^1-piperideine-6-carboxylic acid per hr. The specific activity is defined as units per milligram of protein. Protein in the crude and partially purified enzyme preparations was determined by the procedure of Lowry *et al.* (1951) using egg albumin as standard; with most column fractions, protein elution patterns were estimated by the 280-mμ absorption. Concentrations of the purified enzyme were derived from the absorbance at 280 mμ. A $E^{1\%}_{1\,cm}$ value of 7.35 was used throughout which was obtained by absorbance and dry weight determinations. Absorption spectra were taken with a Shimadzu MPS-50L recording spectrophotometer with a 1-cm light path. The molecular weight of the enzyme was determined by the ultracentrifugal sedimentation equilibrium method according to the procedure of Van Holde and Baldwin (1958). The experiments were carried out in a Spinco Model E ultracentrifuge equipped with Rayleigh interference optics. Multicell operation was employed in order to perform the experiment on five samples of different initial concentration ranging from 0.1 to 0.5%, with the use of An-G rotor and double-sector cells of different side-wedge angles. According to the method of Hexner *et al.* (1961) to shorten the time to attain the equilibrium, the rotor was centrifuged initially at 11,272 rpm for 2 hr and 30 min and then at 9341 rpm. The liquid height was 0.15 cm. Interference patterns were photographed with Fuji panchromatic process plate at intervals of 1 hr to compare and make sure the equilibrium was established. The relation between the concentration of the enzyme and the fringe shift was determined using the synthetic boundary cell. An exposure time of 20–40 sec was sufficient when Fuji Neopan SSS plates were used.

Results

Purification of Enzyme. All operations were carried out at 0–5°, unless specified otherwise.

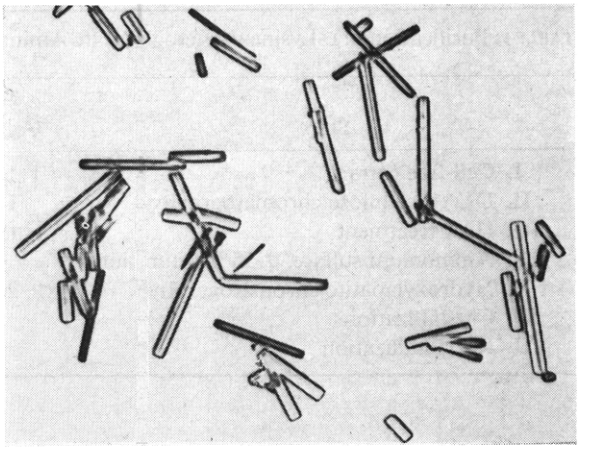

FIGURE 2: Crystals of L-lysine–α-ketoglutarate aminotransferase (× 2000).

STEP I. The washed cells were suspended in 0.1 M potassium phosphate buffer (pH 7.4) containing 10^{-5} M pyridoxal 5′-phosphate and 0.02% 2-mercaptoethanol and disrupted by treatment for 10 min in a 19-kc Kaijo Denki ultrasonic disintegrator. The intact cells and cell debris were removed by centrifugation. The supernatant was dialyzed overnight against 0.01 M potassium phosphate buffer (pH 7.4) containing 10^{-5} M pyridoxal 5′-phosphate and 0.01% 2-mercaptoethanol.

STEP II. The dialyzed enzyme solution was placed on a DEAE-cellulose column (6.0 × 45 cm) equilibrated with the dialysis buffer. After the column was washed thoroughly with the same buffer, the enzyme was eluted stepwise with the buffer supplemented with sodium chloride (final concentration 0.04 and 0.1 M). The enzyme activity was found only in the fractions eluted with the buffer containing 0.1 M sodium chloride. The active fractions were combined and concentrated by addition of ammonium sulfate (60% saturation). The precipitate was dissolved in 0.01 M potassium phosphate buffer (pH 7.4) containing 10^{-4} M pyridoxal 5′-phosphate and 0.02% 2-mercaptoethanol.

STEP III. The enzyme solution was kept at 55° for 5 min, cooled rapidly, and centrifuged at 17,000*g* for 20 min to remove the precipitate. The enzyme was activated by this treatment; the mechanism of such activation is at present unknown.

STEP IV. The supernatant was brought to 55% saturation with ammonium sulfate and centrifuged at 17,000*g* for 30 min. The precipitate obtained was dissolved in a small volume of 0.001 M potassium phosphate buffer (pH 7.2) containing 10^{-5} M pyridoxal 5′-phosphate and 0.01% 2-mercaptoethanol, dialyzed against the same buffer, and centrifuged.

STEP V. The supernatant was applied to a hydroxylapatite column (3.0 × 25 cm) equilibrated with 0.001 M potassium phosphate buffer (pH 7.2) containing 10^{-5} M pyridoxal 5′-phosphate and 0.01% 2-mercaptoethanol. The enzyme was eluted with the same buffer. The elution pattern is shown in Figure 1. The active fractions were collected and concentrated by ammonium sulfate precipitation (60% saturation). The precipitate was dissolved in 0.2 M potassium phosphate buffer (pH 7.2)

TABLE I: Purification of L-Lysine–α-Ketoglutarate Aminotransferase.

Step	Vol (ml)	Total Protein (mg)	Sp Act. (units/mg)	Total Units	Yield (%)
I. Cell-free extract	1,180	39,400	5.96	235,000	100
II. DEAE-cellulose chromatography	127	1,594	117.2	187,000	80
III. Heat treatment	197	625	395.0	247,000	105
IV. Ammonium sulfate, 0–55% saturation	47	537	354.0	190,000	81
V. Hydroxylapatite chromatography	223	110	1,006	111,000	47.2
VI. Crystallization	6.5	85.3	1,100	93,800	40.0
VII. Recrystallization	4.1	42.4	1,120	47,500	20.2

containing 10^{-4} M pyridoxal 5′-phosphate and 0.01% 2-mercaptoethanol.

STEP VI. Ammonium sulfate was added slowly to the enzyme solution until a faint turbidity was obtained. The pH of solution was kept constant at 7.2 with 14% ammonium hydroxide solution. On standing overnight at about 3° crystal formation occurred. The crystals took the form of yellow rods (Figure 2).

STEP VII. The crystalline enzyme was collected by centrifugation and dissolved in a small volume of 0.01 M potassium phosphate buffer (pH 7.2) containing 10^{-4} M pyridoxal 5′-phosphate and 0.01% 2-mercaptoethanol. The enzyme was recrystallized as described in step VI.

The specific activity of the enzyme was not appreciably enhanced by crystallization or recrystallization. Approximately 180-fold purification was achieved with an over-all yield of 20.2%. A summary of the purification procedure is presented in Table I.

Purity and Physicochemical Properties of Enzyme. SEDIMENTATION PROPERTIES. The purity of the crystalline enzyme and its sedimentation coefficient were determined with a Spinco Model E ultracentrifuge equipped with a phase plate as a schlieren diaphragm. As shown in Figure 3, the schlieren patterns obtained indicate the presence of a single component. The sedimentation coefficient of the protein peak, calculated for water at 20° and zero protein concentration, was 6.37 S. The molecular weight of the enzyme was determined by the method of Van Holden and Baldwin (1958) as described above. Enzyme solution at five different concentrations, 1.0, 2.0, 3.0, 4.0, and 5.0 mg per ml, were centrifuged at 20°. The plot of log protein concentration

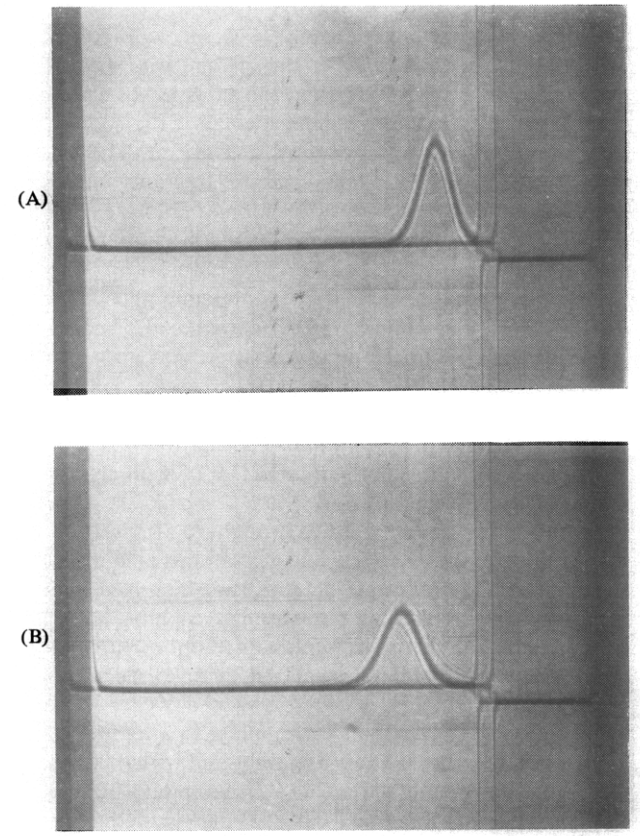

FIGURE 3: Sedimentation pattern of crystalline L-lysine–α-ketoglutarate aminotransferase. Protein concentration, 8 mg/ml in 0.01 M potassium phosphate buffer (pH 7.0). Pictures were taken at bar angle of 80°. (A) 24 min after achieving top speed (42,040 rpm); (B) 56 min later.

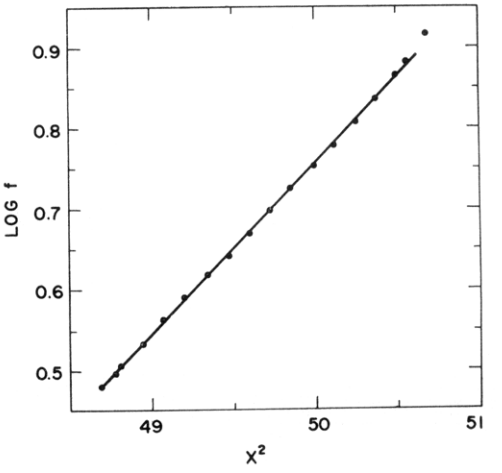

FIGURE 4: Sedimentation equilibrium pattern of L-lysine–α-ketoglutarate aminotransferase. The sample initially contained 5 mg of protein/ml in 0.01 M potassium phosphate buffer (pH 7.0). Log protein concentration is shown as a function of square of the distance from the center of rotor: *f*, fringe shift (millimeters); *X*, the distance from the center of the rotor (centimeters).

TABLE II: Activity of L-Lysine–α-Ketoglutarate Aminotransferase, Resolved, and Reconstituted.[a]

Enzyme Preparation	Relative Activity (%)	
	−Pyridoxal 5′-Phosphate	+Pyridoxal 5′-Phosphate
Before resolution	100	122.5
After resolution	3.1	107.5
After incubation with pyridoxal 5′-phosphate and dialysis	87.5	110
After incubation with pyridoxamine 5′-phosphate and dialysis	82.0	107

[a] The concentration of pyridoxal 5′-phosphate and pyridoxamine 5′-phosphate were 10^{-4} M. The enzyme activity was assayed by procedure B.

against the square of the distance from the center of the rotor is shown in Figure 4. Assuming a partial specific volume of 0.74, a molecular weight of 116,000 ± 3000 was obtained.

ELECTROPHORETIC PROPERTIES. The crystalline enzyme gives a single component when examined by free-boundary electrophoresis (Figure 5). Disc gel electrophoresis in 7.5% polyacrylamide gel was performed by a modification of the method of Davis (1964). The enzyme (29.3 μg) was placed on the top of spacer gel in 1 M sucrose. After the run, protein was stained with 1.0% Amido-Schwarz in 7% acetic acid. Destaining was accomplished in 7% acetic acid. The enzyme is homogeneous by this criterion, as shown in Figure 6.

STABILITY OF ENZYME. The stability of the enzyme depended upon the degree of purification and the conditions. The enzyme in the dialyzed sonicate (step I of purification) was considerably labile to heating at 50°, but when partially purified by DEAE-cellulose column chromatography (step II), it was stable to heating and was activated by standing at 50–60° for 5 min as demonstrated in Figure 7. When a preparation of this aminotransferase was heated at various values of pH, the enzyme was found to be stable between pH 6.0 and 7.5 (Figure 8). Pyridoxal 5′-phosphate (10^{-4} M) and 2-mercaptoethanol (0.02%) afforded good protection of the enzyme activity against heat inactivation.

Enzyme Cofactor Interaction. ABSORPTION SPECTRUM. The spectrum of the enzyme prepared freshly at pH 7.4 is shown in Figure 9. There are three absorption maxima: at 280, 340, and 415 mμ; these give an absorbance ratio of 100:12:11. The molecular extinction coefficients are 85,260 at 280 mμ, 10,230 at 340 mμ, and 9380 at 415 mμ. The absorbance ratio changes gradually during storage of the enzyme. No appreciable spectral shifts occurred on varying pH (5.5–9.5).

Spectral shifts were observed in the presence of the amino donors. The addition of L-lysine to the enzyme

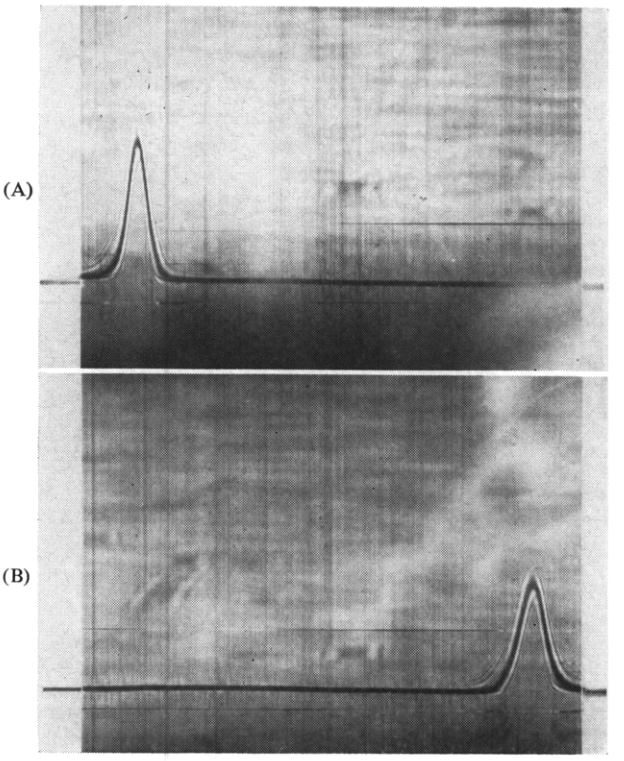

FIGURE 5: Free-boundary electrophoresis pattern of L-lysine–α-ketoglutarate aminotransferase: (A) ascending boundary; (B) descending boundary. The enzyme solution (8.9 mg/ml) was subjected to electrophoresis in a Hitachi (HTB-2A) free-boundary electrophoresis apparatus equipped with a 2-ml cell. The temperature was 4° and sodium phosphate buffer (pH 7.0; ionic strength, 0.2) was employed. A current of 10 mA (56 V) was applied. Pictures were taken at bar angle of 30° at 2 hr after initiation of the experiment. Migration is toward the anode on the right (A) and on the left (B).

solution at pH 7.4 caused a decrease in the absorbance at 415 mμ and an increase in that at about 340 mμ with concomitant shift of the maximum position of the peak from 340 to 330 mμ (Figure 10). A similar effect was observed by addition of L-ornithine; this amino acid is less active as described below, and the spectral change occurred more slowly than with L-lysine. The absorption spectrum, however, was not influenced substantially by addition of α-ketoglutarate at pH 7.4.

RESOLUTION AND RECONSTITUTION. Pyridoxal 5′-phosphate was required for maximal activity of the enzyme. Pyridoxal 5′-phosphate may be partially removed from the native enzyme by dialysis. Exhaustive dialysis of the enzyme caused a decrease in catalytic activity which could be restored by addition of pyridoxal 5′-phosphate. Table II (the first line) shows that the holoenzyme prepared and dialyzed as usual is approximately 20% resolved.

In the absence of added pyridoxal 5′-phosphate and α-ketoglutarate, L-lysine at moderate concentration (5–20 mM; at pH 7.4 or 8.0) rapidly inactivated L-lysine–α-ketoglutarate aminotransferase with associated spectral shifts as shown in Figure 10; such inactivation was prevented by adding pyridoxal 5′-phosphate.

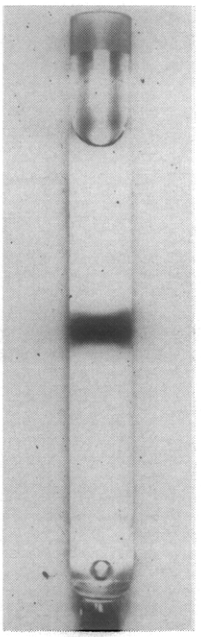

FIGURE 6: Disc gel electrophoresis of L-lysine–α-ketoglutarate aminotransferase. Electrophoresis was conducted at a current of 2 mA for 2 hr in Tris–glycine buffer (pH 9.0). The direction of migration is from the cathode (top of photo) to the anode. Other conditions are given in text.

Resolution of the aminotransferase was carried out by incubation of the enzyme with L-lysine essentially as described by Scardi *et al.* (1963). Thus, the enzyme (5.02 mg) was incubated (37°, pH 7.4, 30 min) with 40 μmoles of L-lysine in a final volume of 0.6 ml. After addition of 1.2 ml of 1 M potassium dihydrogen phosphate (pH 5.0), the mixture was kept at 37° for another

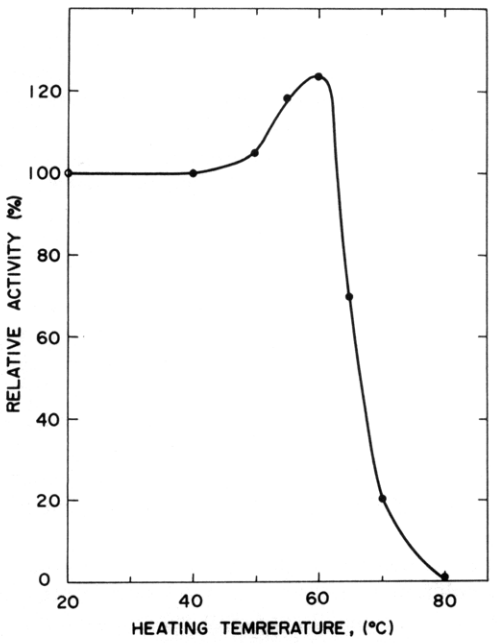

FIGURE 7: Effect of temperature on the enzyme stability. The enzyme preparation was heated at the indicated temperature for 5 min. The enzyme activity was assayed by procedure B.

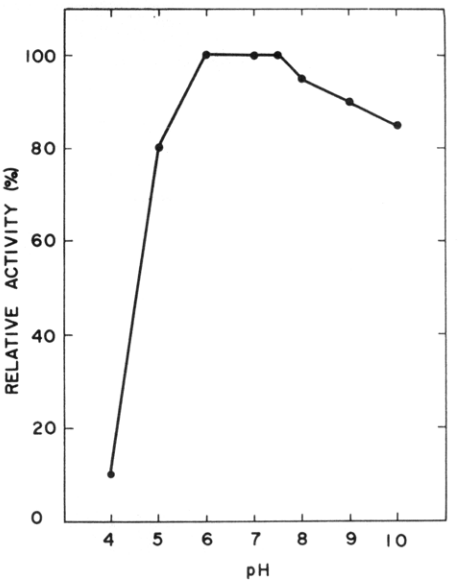

FIGURE 8: Effect of pH on the enzyme stability. The enzyme preparation was heated at 55° for 5 min at the indicated pH. The enzyme activity was assayed by procedure B.

30 min. Ammonium sulfate was added to bring the mixture to 60% saturation. The resulting precipitate was dissolved in a small volume of 0.01 M potassium phosphate buffer (pH 7.4) and dialyzed overnight against the same buffer. The enzyme thus treated had almost no detectable activity in the absence of added pyridoxal 5′-phosphate, although substantially full activity was obtained in its presence (Table II). This suggests that the aminotransferase was resolved to an inactive form by incubation with L-lysine. This inactive form of the enzyme was designated "semiapoenzyme," because it

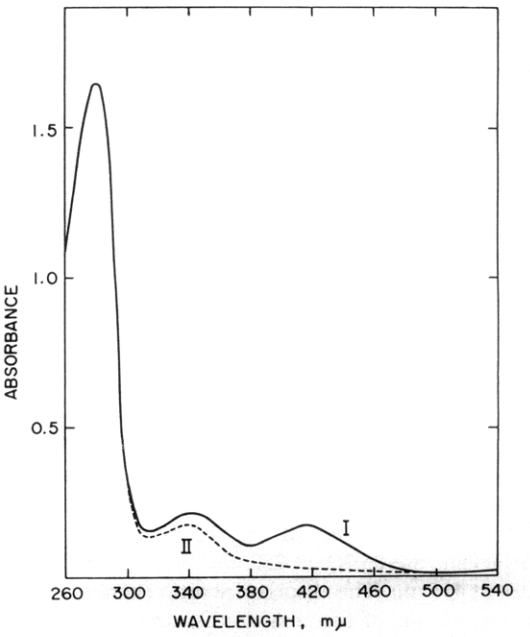

FIGURE 9: Absorption spectra of L-lysine–α-ketoglutarate aminotransferase (2.23 mg/ml) in 0.01 M potassium phosphate buffer (pH 7.4). (I) Holoenzyme; (II) semiapoenzyme.

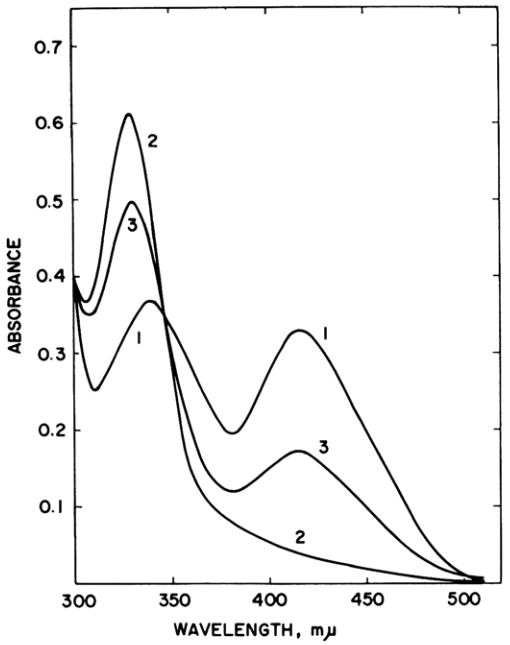

FIGURE 10: Absorption spectra of L-lysine–α-ketoglutarate aminotransferase in the presence of amino donors. Absorption spectra were determined after incubation of the enzyme (1.16 mg/ml) in 0.2 M potassium phosphate buffer (pH 7.2) with amino donors at 30° for 20 min. Additions: curve 1, none; curve 2, 5 mM L-lysine; curve 3, 10 mM L-ornithine.

still contains 1 mole of pyridoxal 5′-phosphate bound tightly/mole of protein as described below. The semiapoenzyme was crystallized as follows. Ammonium sulfate was added slowly until the solution became slightly turbid. On standing overnight at about 3°, the protein precipitated as crystalline rods (Figure 11). The dialyzed inactive form of enzyme exhibited no peak at 415 mμ and a decreased peak around 330 mμ with an absorption maximum at 340 mμ (Figure 9). The semiapoenzyme was then incubated with pyridoxal 5′-phosphate and dialyzed against 0.01 M potassium phosphate buffer (pH 7.4). As indicated in Table II, the holoenzyme can be obtained from the semiapoenzyme by addition of pyridoxal 5′-phosphate. Pyridoxal 5′-phosphate could be replaced by pyridoxamine 5′-phosphate, but neither by pyridoxal nor pyridoxamine. The Michaelis constants for pyridoxal 5′-phosphate and pyridoxamine 5′-phosphate were estimated to be 3.57×10^{-7} and 7.15×10^{-6} M, respectively (Figure 12).

PYRIDOXAL 5′-PHOSPHATE ANALYSIS. Since pyridoxal 5′-phosphate is gradually released from the holoenzyme on dialysis or during storage, the enzyme in 0.01 M potassium phosphate buffer (pH 7.4), containing 10^{-4} M pyridoxal 5′-phosphate, was dialyzed overnight against 0.01 M potassium phosphate buffer (pH 7.4) containing 2×10^{-5} M pyridoxal 5′-phosphate in order to avoid partial resolution.

The vitamin B_6 content of the enzyme was determined in duplicate experiments using the two different enzyme samples according to the following four independent methods by deducting the contents in the dialysate from those in the dialyzed enzyme.

Phenylhydrazine Method. After the dialyzed enzyme was kept at room temperature for 30 min in the presence of 0.1 N HCl to release the bound cofactor, the amount of free pyridoxal 5′-phosphate was determined with phenylhydrazine reagent (Wada and Snell, 1961). Average value of 1 mole of pyridoxal 5′-phosphate/54,200 g of protein was obtained.

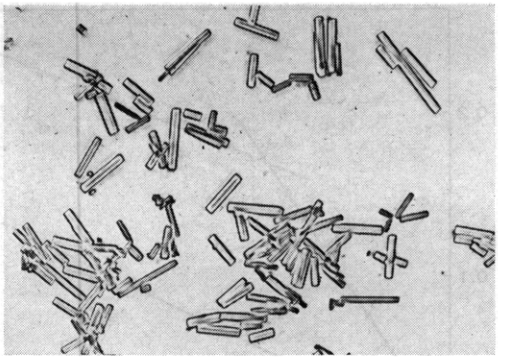

FIGURE 11: Crystals of semiapoenzyme (× 1200).

Microbioassay. The enzyme samples were hydrolyzed with acid (Rabinowitz and Snell, 1947) and assayed microbiologically for vitamin B_6 (Snell, 1950). The averaged value of 1 mole of vitamin B_6/55,600 g of protein was obtained.

Cyanohydrin Method. The enzyme samples were hydrolyzed with acid (Rainowitz and Snell, 1947) and analyzed by the cyanohydrin method of Bonavita (1960). An average vitamin B_6 content of 1 mole/55,500 g of protein was obtained.

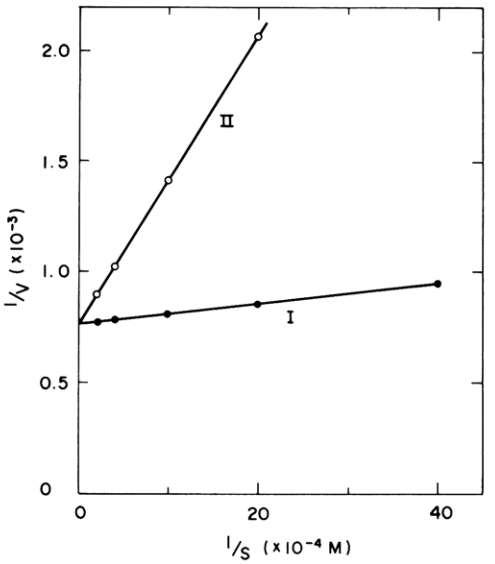

FIGURE 12: Effect of pyridoxal 5′-phosphate and pyridoxamine 5′-phosphate on the activity of semiapoenzyme. The reaction mixture consisted of 40 μmoles of L-lysine, 40 μmoles of potassium α-ketoglutarate, 90 μmoles of potassium phosphate buffer (pH 8.0), the indicated concentration of pyridoxal 5′-phosphate or pyridoxamine 5′-phosphate, and 13 μg of semiapoenzyme in a final volume of 2.0 ml. Incubation was carried out at 37° for 10 min. The enzyme activity was assayed by procedure B. The reciprocal velocity was plotted against the reciprocal concentration of pyridoxal 5′-phosphate (I) or pyridoxamine 5′-phosphate (II).

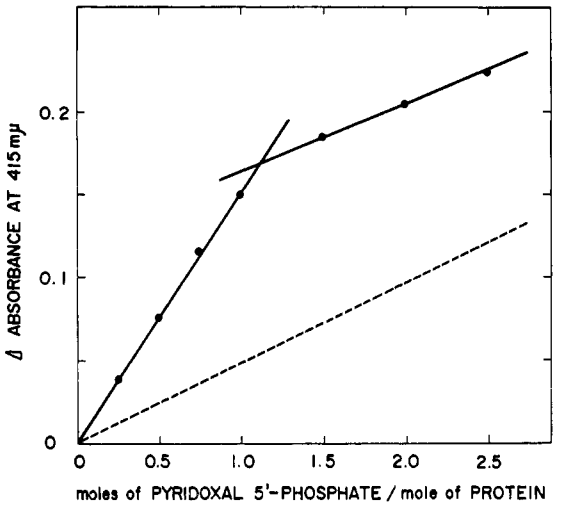

FIGURE 13: Spectrophotometric titration curve of semiapoenzyme with pyridoxal 5′-phosphate. Pyridoxal 5′-phosphate was added to a solution of the semiapoenzyme (2.125 mg/ml) in 0.1 M potassium phosphate buffer (pH 7.2) to give the following ratios of pyridoxal 5′-phosphate (mole) to protein: 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, and 2.5. Absorption spectrum was measured at 25°. The difference between the absorbance at 415 mμ of the semiapoenzyme plus pyridoxal 5′-phosphate and the absorbance of the semiapoenzyme is plotted against the moles of pyridoxal 5′-phosphate added per mole of enzyme. The dotted line gives the absorbance at 415 mμ when equivalent amounts of pyridoxal 5′-phosphate were added to a control in which enzyme was omitted.

TABLE III: Amino Donor Specificity.

Amino Donor[a]	Rel Act. (%)
L-Lysine	100
δ-Hydroxylysine (DL-, DL-*allo*-)[b]	0
α-*N*-Acetyl-L-lysine	0
ε-*N*-Methyl-L-lysine	0
ε-*N*-Acetyl-L-lysine	0
L-Ornithine	54.5
L-α,γ-Diaminobutyrate	0
ε-Aminocaproate	0
δ-Aminovalerate	0
γ-Aminobutyrate	0
DL-Norleucine[c]	0
DL-Norvaline[c]	0
L-Leucine	0
L-Valine	0
Cadaverine	0
Putrescine	0
L-Arginine	0
L-Phenylalanine	0
L-Aspartate	0

[a] The concentration of the amino donor: 10 mM, [b] 20 mM, and [c] 40 mM. The enzyme activity was assayed by procedure A.

Spectrophotometric Titration with Pyridoxal 5′-Phosphate. The peak at 340 mμ was found to remain even in the catalytically inactive form (semiapoenzyme), although the one at 415 mμ disappeared as described above (Figure 9). The semiapoenzyme samples were analyzed for vitamin B_6 by the microbioassay and by the cyanohydrin method. The vitamin B_6 content of this form was 1 mole of vitamin B_6/102,500 g of protein as determined by the microbiological assay, and 1 mole/124,400 g of protein as determined by the cyanohydrin method.

The spectrum of the enzyme was recorded in an experiment in which the semiapoenzyme was treated with increasing amount of pyridoxal 5′-phosphate. The absorbance at 415 mμ minus the absorbance of the semiapoenzyme at this wavelength was plotted against the moles of pyridoxal 5′-phosphate added per mole of enzyme (116,000). This curve changes slope sharply after about 1.1 moles of pyridoxal 5′-phosphate/mole of enzyme had been added as shown in Figure 13. The slope after the sharp break is close to that of a control in which enzyme was omitted. Although free pyridoxal 5′-phosphate exhibits maximum absorbance at 388 mμ, it exhibits appreciable absorbance at 415 mμ; therefore, the curve shown in Figure 13 reflects the presence of both enzyme-bound and free pyridoxal 5′-phosphate. The dotted line represents the absorbance of free pyridoxal 5′-phosphate.

The results obtained by these four procedures indicate that 2 moles of pyridoxal 5′-phosphate are bound to 1 mole of enzyme protein in the holoenzyme and that the catalytically inactive semiapoenzyme contains approximately 1 mole of pyridoxal 5′-phosphate/mole of enzyme protein.

Substrate Specificity. The ability of this aminotransferase to catalyze the amino group transfer from various amino donors to α-ketoglutarate is presented in Table III. In addition to L-lysine, L-ornithine showed approximately 55% reactivity under the conditions employed in comparison with L-lysine, although the optimum pH for L-ornithine–α-ketoglutarate transamination is about 7.5 as mentioned below. No activity was observed with the other amino acids and amines tested. The transamination product from L-ornithine reacted with *o*-aminobenzaldehyde and ninhydrin to develop an orange and a yellow color, respectively, in the same manner as reported on the product from L-lysine (Soda *et al.*, 1968). Authentic Δ^1-pyrroline-2-carboxylic acid was prepared by the oxidation of D-proline with D-amino acid oxidase prepared from pig kidney in order to compare it with the product from L-ornithine. Authentic Δ^1-piperideine-2-carboxylic acid was prepared as described by Meister (1954). On paper electrophoresis, the transamination product from L-ornithine was separated satisfactorily from Δ^1-pyrroline-2-carboxylic acid and Δ^1-piperideine-2-carboxylic acid and showed properties similar to Δ^1-piperideine-6-carboxylic acid as shown in Figure 14. Although the isolation of the product and its further investigation were not performed, these findings suggest that the terminal amino group of L-ornithine is transaminated to α-ketoglutarate to yield Δ^1-pyrroline-5-carboxylic acid. This compound

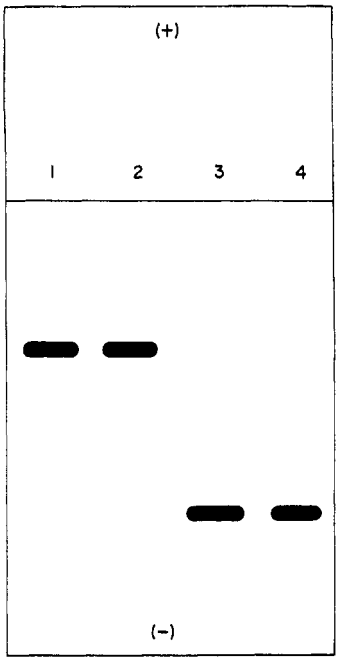

FIGURE 14: Paper electrophoresis of the transamination product from L-ornithine. Electrophoresis was carried out in 1 N formic acid at 3000 V for 1 hr. (1) Δ^1-Piperideine-2-carboxylic acid, (2) Δ^1-pyrroline-2-carboxylic acid, (3) the transamination product from L-ornithine, and (4) Δ^1-piperideine-6-carboxylic acid. Spots were visualized by development with *o*-aminobenzaldehyde.

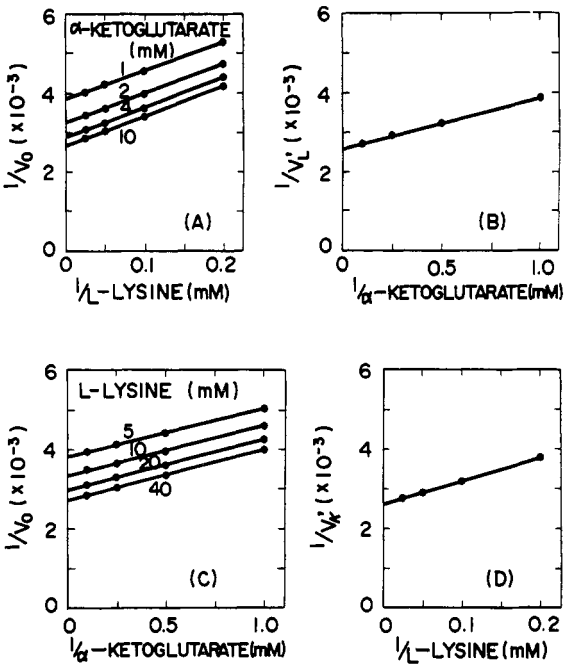

FIGURE 15: Determination of the Michaelis constants for L-lysine and α-ketoglutarate. The reaction mixture contained 1 μmole of pyridoxal 5′-phosphate, 90 μmoles of potassium phosphate buffer (pH 8.0), and variable amounts of the substrates as indicated in the figure. The enzyme activity was assayed by procedure B. (A) Double-reciprocal plots of initial velocity, V_0, against L-lysine concentration at a series of fixed concentrations of α-ketoglutarate; (B) secondary plot from the intercepts, $1/V_L'$, of A; (C) double-reciprocal plots of V_0 against α-ketoglutarate concentration at a series of fixed concentrations of L-lysine; (D) secondary plot from the intercepts $1/V_K'$ of C.

must be formed from glutamate-γ-semialdehyde which is produced directly from L-ornithine by the aminotransferase reaction with α-ketoglutarate.

α-Ketoglutarate was the only active amino acceptor for L-lysine and L-ornithine. No activity was obtained with glyoxylate, pyruvate, oxaloacetate, α-ketobutyrate, α-ketovalerate, and α-ketocaproate.

Kinetics. The Michaelis constants for L-lysine and α-ketoglutarate were determined according to the method of Velick and Vavra (1962). They were calculated to be 2.8×10^{-3} M for L-lysine and 5.0×10^{-4} M for α-ketoglutarate, as shown in Figure 15. The estimated maximum turnover number is 2200 moles/min per mole of enzyme. When L-ornithine was employed as an amino donor, the K_m values for L-ornithine and α-ketoglutarate were 2.0×10^{-3} and 1.3×10^{-4} M, respectively.

Effect of pH. The enzyme when examined in the presence of potassium phosphate, Veronal, and borate buffers has an optimum reactivity in the pH range of 8.3–8.5 for L-lysine–α-ketoglutarate transamination and at pH 7.5 for L-ornithine–α-ketoglutarate transamination as shown in Figure 16. Tris–HCl buffer (pH 8.0) inhibited the enzyme by approximately 25%.

Discussion

L-Lysine–α-ketoglutarate aminotransferase has been purified 180-fold and crystallized from the crude extracts of *A. liquidum* IFO 3084. The enzyme can also be purified from *F. fuscum* AKU 0140 and *F. flavescens* AKU 0141 in lower yield. The crystalline enzyme is homogeneous by the criteria of both disc gel and free-boundary electrophoresis and also by ultracentrifugation. The visible spectrum of crystalline enzyme is characterized by maxima at 340 and 415 mμ. The shape of the spectrum of L-lysine–α-ketoglutarate aminotransferase closely resembles those of other aminotransferases, *e.g.*, D-alanine–D-glutamate aminotransferase (Martinez-Carrion and Jenkins, 1965), L-leucine aminotransferase (Taylor and Jenkins, 1966), L-alanine aminotransferase (Saier and Jenkins, 1967), and L-tyrosine aminotransferase (Hayashi *et al.*, 1967). L-Lysine–α-ketoglutarate aminotransferase behaves like the L-leucine aminotransferase and D-alanine–D-glutamate aminotransferase in that the spectra of these enzymes do not appear to change with pH. However, the spectra of L-glutamate–L-aspartate aminotransferase (Jenkins and Sizer, 1959) and L-alanine aminotransferase (Saier and Jenkins, 1967) are shifted on varying pH. Addition of either L-lysine or L-ornithine to the holoenzyme caused a decrease in absorbance at 415 mμ and an enhancement in that at about 340 mμ with simultaneous shift of the absorption maximum from 340 to 330 mμ. Exhaustive dialysis of the enzyme treated with the amino donor resulted in the return of the absorption peak from 330 to 340 mμ with a decrease in absorbance. The absorption maximum at 415 mμ, by analogy with other aminotransferases, may be attributed

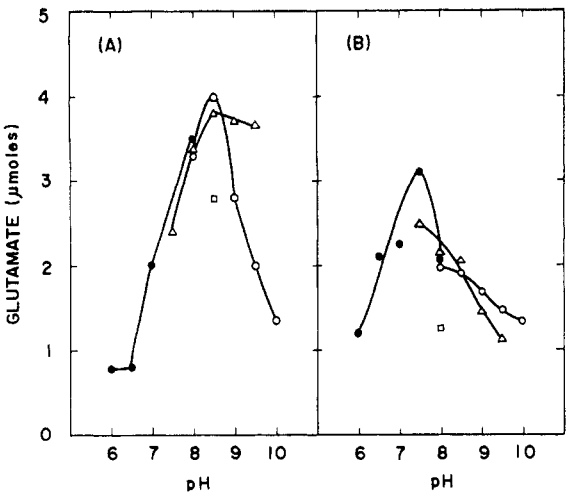

FIGURE 16: Effect of pH on L-lysine–α-ketoglutarate transamination (A) and L-ornithine–α-ketoglutarate transamination (B). The reaction mixture contained 20 μmoles of L-lysine or L-ornithine, 20 μmoles of α-ketoglutarate, 1 μmole of pyridoxal 5′-phosphate, and 90 μmoles of the following buffer in a final volume of 2.0 ml: (●—●) potassium phosphate, pH 6.0–8.0; (△—△) Veronal, pH 7.5–9.5; (○—○) borate, pH 8.0–10.0; (□) Tris–HCl, pH 8.0. The enzyme activities were assayed by procedure A.

to a protonated aldimine formed between pyridoxal 5′-phosphate and an amino group of enzyme. The absorbance at 330 mμ could be ascribed to the phosphopyridoxamine form, which has been shown to be less firmly bound to the apoenzyme (Wada and Snell, 1962; Scardi *et al.*, 1963). When α-ketoglutarate was added to the holoenzyme having the absorption maxima at 340 and 415 mμ, no appreciable change in the absorption spectrum was observed.

The enzyme resolved by incubation with L-lysine in the presence of phosphate ions is catalytically inactive and retained a peak at 340 mμ even after dialysis. The activity of the inactive form of enzyme was not restored with pyridoxal and pyridoxamine, but with pyridoxal 5′-phosphate and pyridoxamine 5′-phosphate.

The vitamin B_6 derivative contained in the holoenzyme reacts with phenylhydrazine and cyanohydrin, and 2 moles of vitamin B_6 were found to be bound to 1 mole of enzyme protein. The vitamin B_6 analyses of the inactive form of enzyme by the microbiological and cyanohydrin methods show that 1 mole of this resolved enzyme still contains 1 mole of the bound pyridoxal 5′-phosphate, from which the absorbance at 340 mμ was derived. This half-resolved form of the enzyme is regarded as a new form of enzyme, *i.e.*, "semiapoenzyme." It is suggested that the catalytic activity of holoenzyme is concerned with only 1 mole of the bound pyridoxal 5′-phosphate with the absorption peak at 415 mμ which was converted into the pyridoxamine 5′-phosphate with the absorption maximum at 330 mμ by the treatment with the amino donor. The bound pyridoxal 5′-phosphate with the absorption peak at 340 mμ seems not to participate in the catalytic action of the enzyme. Although this absorption may be due to tautomerization (Heinert and Martell, 1963) and to the addition of NH, SH, or OH across the imine double bond formed between the protein moiety of enzyme and the cofactor (Kent *et al.*, 1958; Fischer *et al.*, 1958; Dempsey and Christensen, 1962), further work is needed to elucidate the binding of cofactor.

The terminal amino group of L-ornithine is transferred more slowly to α-ketoglutarate by this aminotransferase than that of L-lysine. It was reported that L-ornithine–δ-aminotransferase from rat liver cannot catalyze the transamination of L-lysine with α-ketoglutarate (Strecker, 1965). L-Lysine–α-ketoglutarate aminotransferase differs unequivocally from L-ornithine–δ-aminotransferase. Since the enzyme cannot catalyze the transamination of α-*N*-acetyl-L-lysine and ε-aminocaproate, α-amino group of L-lysine is required to be free in this ε transamination. The role played by the α-amino group in the reaction mechanism remains unsettled.

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Studies on Δ5→4-3-Oxo Steroid Isomerases. I. An Extraction Model for Enzymatic Activity*

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ABSTRACT: *Pseudomonas testosteroni* Δ5→4-3-oxo steroid isomerase has been tested for analyzing the enzyme–substrate or inhibitor complex formation in terms of an extraction process of steroid from the aqueous medium by the hydrophobic part of the protein. 5-Androstene-3,17-dione, 5-estrene-3,17-dione, and 5-pregnene-3,20-dione have been used as substrates, and 19-nortestosterone, 4-estrene-3,17-dione, progesterone, and 19-norprogesterone as competitive inhibitors, varying the methanol concentration of the reaction medium. Log $1/K_m$ and log $1/K_i$, according to the case, plotted *vs.* methanol concentration, decrease linearly. Log K_p (partition coefficient of the steroids between isooctane and various methanol–water mixtures) *vs.* methanol concentration decreases also linearly. These results are in favor of the extraction model.

Parallel straight lines are obtained with a given steroid for $1/K_m$ or log $1/K_i$ *vs.* log K_p, which indicate the participation of the hydrophobic groups of steroids to the enzyme–substrate binding. The V_{max} increase induced by methanol for 5-estrenedione and 5-pregnenedione isomerization (but not for 5-androstenedione) is discussed in terms of relative affinity of substrate and reaction product in each case.

Various 19-nor-Δ4-3-oxo steroids have been used at a given methanol concentration. Among the structural features of the steroid molecule which interfere in steroid–protein interaction, the substitution of the C_{19} methyl group by hydrogen could increase the affinity by favoring enol formation.

The Δ5→4-3-oxo steroid isomerase of *Pseudomonas testosteroni* (EC 5.3.3.1.)[1] provides a rather simple model of direct interaction between a steroid molecule and a protein. This enzyme, studied by Talalay, requires no prosthetic group and the isomerization reaction occurs by intramolecular hydrogen transfer without incorporation of protons from the medium (Kawahara *et al.*, 1962; Wang *et al.*, 1963).

Steroid substrates are rigid hydrocarbon molecules of low solubility and the formation of enzyme–substrate complex may largely involve hydrophobic interactions. As changes in the structure of water surrounding the nonpolar groups play a major role in these interactions, the nature of the solvent medium appears to be of particular importance.

Changes in composition of the reaction medium and their effects on enzymatic reactions have been extensively studied and give some information on the nature of enzyme–substrate interactions. A number of water-

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[1] Isomerase will be used in this paper.