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FURTHER CHARACTERIZATION OF L-LEUCINE-PYRUVATE TRANSAMINASE FROM *ACETOBACTER SUBOXYDANS*

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Summary

L-Leucine-pyruvate transaminase obtained from *Acetobacter suboxydans* exhibited absorbance maxima at 280 and 332 nm. The 332 nm peak was derived from the coenzyme bound to the enzyme protein with the ϵ-NH_2 of a lysine residue.

The transaminase showed reactivity against many L-amino acids. The relation between the reactivity and the structure of the amino donor is discussed. The Michaelis constants for L-leucine, pyruvate, L-alanine and α-ketoisocaproate were 6.7, 3.1, 7.1 and 0.9 mM, respectively. The equilibrium constant was 5.3. The activation energy at pH 5.0 was 8,800 cal/mol.

Introduction

In previous papers [1,2], we have reported evidence for the occurrence of a transamination of L-leucine with pyruvate in the cell-free extracts of *Acetobacter suboxydans (Gluconobacter suboxydans* IFO 3172). The purification method and some characteristics of L-leucine-pyruvate transaminase (tentative name) were also investigated [3]. The distinctive features of the enzyme were (1) acidic optimum pH, (2) unusual absorbance spectrum and (3) broad substrate specificity. The present paper describes the results of detailed experiments about points (2) and (3) and other properties.

Materials

L-Leucine-pyruvate transaminase was prepared from cell extracts of *A. suboxydans* by the procedure described in the previous paper [3]. When the enzyme preparation was examined by analytical ultracentrifugation, all protein

migrated as a single component ($S^0_{20,w}$, 5.23 S). Electrophoresis on cellulose acetate strips in potassium phosphate buffer (pH 6.1) also revealed the presence of a single protein in the preparation.

Various amino acids used in this work were kindly supplied by Drs T. Yamamoto and K. Soda, Institute for Chemical Research, Kyoto University. α-Ketoisocaproate and α-keto-δ-guanidinovalerate were prepared according to the method of Meister [4,5]. Lactate dehydrogenase was purchased from Boehringer Mannheim. The other chemicals were commercial products.

Methods

Enzyme assay. The reaction mixture (per ml) contained 50 μmol of L-leucine and pyruvate, 0.1 μmol of pyridoxal 5′-phosphate, 100 μmol of acetate buffer (pH 5.0) and 0.3—1.0 μg of the enzyme protein. After incubation at 37°C, the reaction was stopped by immersing the tube in boiling water for 3 min. The enzyme activity was measured by determining the amount of L-alanine by ninhydrin colorimetry after paper chromatography [1] or that of α-ketoisocaproate according to the method of Tayler and Jenkins [6]. In the reverse reaction, the amount of pyruvate formed was determined enzymatically with lactate dehydrogenase [7].

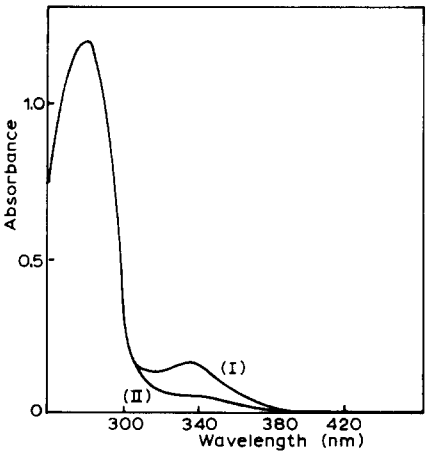
Protein determination. Protein concentration was determined spectrophotometrically by measuring the absorbance at 280 nm ($E^{1\%}_{1\,cm}$, 14.6) [3].

Spectrophotometric measurement. Spectrophotometric measurements were carried out with a Shimadzu MPS-50L recording spectrophotometer. Fluorescence spectra were measured with a Hitachi spectrofluorophotometer Type MPF-2.

Results and Discussion

Absorbance spectrum. The holo-L-leucine-pyruvate transaminase contained 1 mol of pyridoxal phosphate in 1 mol of the enzyme protein [3] and exhibited absorbance maxima at 280 and 332 nm (Fig. 1). No absorbance change was observed when L-leucine was added. The absorbance spectrum was extremely different from those of other vitamin B-6 enzymes [8], especially transaminases.

The apoenzyme, which no longer showed the absorbance maximum at 332 nm, was obtained by incubating the enzyme with 5 mM hydroxylamine at 25°C for 15 min and dialysing against 10 mM potassium phosphate buffer (pH 6.0) for 24 h. The addition of pyridoxal phosphate to the apoenzyme resulted in the full restoration of the activity, with reappearance of the 332 nm peak (PLP-enzyme). The PMP-enzyme which was formed by the incubation of the apoenzyme with 1 mM of pyridoxamine 5′-phosphate at 37°C for 10 min, followed by dialysis against 10 mM potassium phosphate buffer (pH 8.0) containing 10 μM pyridoxamine phosphate, showed the same spectrum as the PLP-enzyme. Judging from these results, it was reasonable that no change of the spectrum was observed on the addition of the amino donor. The fluorescent spectra were also the same in the PLP- and PMP-enzyme (excitation maximum at 332 nm, emission maximum at 384 nm, Fig. 2).



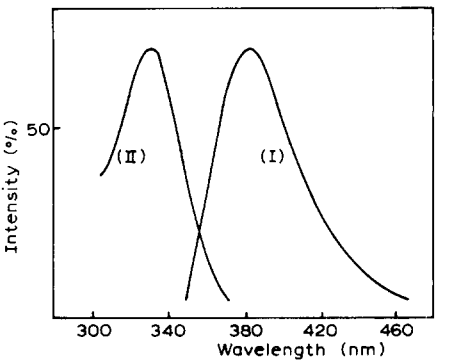


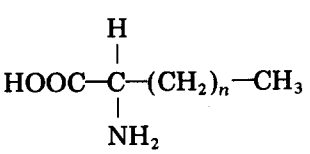
Fig. 1. Absorbance spectra of L-leucine-pyruvate transaminase. Curve I, the enzyme solution (0.82 mg/ml) dialyzed against 10 mM potassium phosphate buffer (pH 6.2) containing 10 μM pyridoxal phosphate; curve II, the enzyme solution dialyzed against the same buffer without pyridoxal phosphate. The spectra were taken against the buffer blank which was used for dialysis.

Fig. 2. Fluorescence spectra of L-leucine-pyruvate transaminase. The enzyme solution (0.91 mg/ml) in 10 mM potassium phosphate buffer (pH 8.0) containing 10 μM pyridoxal phosphate was used. Emission spectrum (I) was measured by exciting at 332 nm and excitation spectrum (II) was measured by analyzing at 384 nm.

Reduction of the enzyme. The PLP-enzyme was treated with 5 mM sodium borohydride at 0°C for 10 min according to the dialysis method of Matsuo and Greenberg [9], and was then dialyzed for 24 hr against 10 mM potassium phosphate buffer (pH 7.0). The reduced enzyme also showed the absorbance maximum at about 330 nm which was never lost during dialysis. It was hydrolyzed for 6 h at 120°C with 6 M HCl. ϵ-*N*-Pyridoxyl lysine was detected and identified in the hydrolysate by paper chromatography and high voltage paper electrophoresis [10].

Thus, all the evidence suggested that absorption at 332 nm was derived from the coenzyme bound at the ϵ-amino group of a lysine residue of the enzyme protein as indicated in other vitamin B-6 enzymes [8].

Substrate specificity. Many L-amino acids served as the amino donor for pyruvate [3]. As shown in Table I, the minimum structural requirement for amino donor seemed to be



(where n = 1—3), and the reactivity was much influenced by the substituted (especially basic and acidic) moiety at the terminal methyl group. The effect was decreased by the presence of methylene group(s) between the α-carbon atom and the terminal group.

TABLE I

AMINO DONOR SPECIFICITY OF L-LEUCINE-PYRUVATE TRANSAMINASE

The concentration of amino donor was changed to 20 mM(*), 10 mM(**) or 100 mM(***).

Amino donor	Relative activity (%)	Amino donor	Relative activity (%)
Glycine	0	L-α-Aminovalerate	100
L-Alanine	—	L-Arginine	48
L-Serine	0	L-Nitroarginine	85
L-Cysteine*	3	L-Citrulline	101
L-Guanidinoalanine	0	L-Ornithine	19
L-α,β-Diaminopropionate	0	α-*N*-Acetyl L-ornithine	0
L-Aspartate	37	L-α-Aminoadipate	65
L-Asparagine	38		
L-Valine	16	L-α-Aminocaproate	91
L-Isoleucine	12	L-Homoarginine	52
L-Phenylalanine	87	L-Homocitrulline	108
L-Tyrosine**	50	L-Lysine	46
L-Histidine	22	L-α-Aminopimelate	84
L-Tryptophan	38	L-α-ε-Diaminopimelate	24
α-Aminoisobutyrate	0	ε-*N*-Methyl L-lysine	69
		α-*N*-Acetyl L-lysine	0
L-α-Aminobutyrate	80	*S*-Aminoethyl L-cysteine	1
L-Homoserine	57		
L-Homocysteine	10	β-Alanine	0
L-Methionine	117	γ-Aminobutyrate	0
DL-Ethionine***	88	ε-Aminocaproate	0
L-Glutamate	52		
L-Glutamine	71	D-Leucine	0
γ-Methyl L-Glutamate	105	D-Methionine	0
DL-Theanine***	75	D-Arginine	0
L-Leucine	100	γ-Methyl D-glutamate	0
γ-Methyl L-leucine	0		
		2-Amino 4-phosphonobutyrate	0
		2-Aminoethylphosphonate	0
		Cadaverine	0
		Taurine	0
		Putrescine	0
		Agmatine	0

α-Ketoglutarate, α-keto-δ-guanidinovalerate or glyoxylate also showed some reactivity as an amino acceptor for L-leucine (Table II).

Recently, Cooper and Meister reported the purification and properties of

TABLE II

AMINO ACCEPTOR SPECIFICITY

Amino acceptor	Relative activity (%)
Pyruvate	100
α-Ketoglutarate	59
Glyoxylate	60
α-Keto-δ-guanidinovalerate	41
Oxalacetate	10

TABLE III

KINETIC CONSTANTS OF L-LEUCINE-PYRUVATE TRANSAMINASE

The reactions were carried out at the standard conditions. The Michaelis constants were obtained by the method of Velick and Vavra [12]. The equilibrium constant was calculated according to the Haldane equation.

K_m value (mM)*				V (mM/30 min)*		Equilibrium value*	Activation energy (cal/mol)**
L-Leucine	Pyruvate	L-Alanine	α-Ketoisocaproate	Forward reaction	Reverse reaction		
6.7	3.1	7.1	0.9	2.38	0.56	5.3	8800

* Enzyme concn, 0.26 μg/ml; incubation, 30 min.
** Enzyme concn, 2.6 μg/ml; incubation, 10 min.

glutamine transaminase in rat liver and demonstrated that the enzyme also shows wide substrate specificity towards amino donors and acceptors [11]. The glutamine transaminase and L-leucine-pyruvate transaminase resemble each other in having broad substrate specificity, but differ distinctly in their enzymatic properties.

Kinetics. The kinetic constants are summarized in Table III. The equilibrium constant calculated according to the Haldane equation [12,13] was nearly in accord with an experimental value of 4.7. The equilibrium values for transaminations between other L-amino acids and pyruvate were almost the same as that of the reaction between L-leucine and pyruvate.

The enzyme showed a maximum reaction velocity at 60—65°C. Over 65°C, the velocity decreased rapidly.

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