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BRANCHED CHAIN AMINO ACIDS AS ACTIVATORS OF BRANCHED CHAIN KETOACID DEHYDROGENASE

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SUMMARY

During purification of branched chain ketoacid dehydrogenase from Pseudomonas putida, large losses in enzyme activity occurred. Much of the activity was restored by the addition of a heat-treated, soluble fraction. The factor had a molecular weight less than 1000 and, of several potential effectors tested, the branched chain amino acids were the only compounds which stimulated enzyme activity, with valine being the most effective. The concentration of valine in the heat-treated fraction was found to be sufficient to account for all of the stimulation produced by this fraction. Valine had no effect on either pyruvate or 2-ketoglutarate dehydrogenases.

Branched chain ketoacid dehydrogenase is a key enzyme in the metabolism of the branched chain amino acids in Pseudomonas, where it is part of a common pathway for the metabolism of valine, isoleucine and leucine (1). The enzyme is under separate genetic control in P. putida (2) and a mutation affecting the enzyme results in loss of ability to oxidize 2-ketoisovalerate, 2-ketoisocaproate and 2-keto-3-methylvalerate but does not affect the oxidation of pyruvate or 2-ketoglutarate (3). Branched chain ketoacid dehydrogenase is presumed to be a complex of decarboxylase, transacetylase and dihydrolipoyl dehydrogenase similar to pyruvate and 2-ketoglutarate dehydrogenases (4,5). The rare, but fatal, human genetic disease, maple syrup urine disease, is characterized by accumulation of these three branched chain ketoacids in serum caused by a defective branched chain ketoacid dehydrogenase (6) with a lesion in either the decarboxylase or transacetylase. There have been some reports of partially purified branched ketoacid dehydrogenases from bovine liver (7) and bacteria (8,9) but none have been purified to the extent that a study of the molecular structure would have been possible. During the purification of branched chain ketoacid dehydrogenase from P. putida, we encountered large losses of enzyme activity which could be restored by a soluble, heat-stable factor. We present evidence in this paper that the soluble factor which stimulates activity of P. putida branched chain ketoacid dehydrogenase is valine.

METHODS

Organism and Growth Conditions. Pseudomonas putida, strain PpG2, was grown with valine as the carbon and energy source as described in previous publications (2, 10, 11).

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Enzyme Assays. The standard assay for branched chain 2-ketoacid dehydrogenase contained 100 μmoles potassium phosphate buffer, pH 7.85; 2 μmoles NAD; 0.5 μmole thiamine pyrophosphate; 0.5 μmole coenzyme A; 1.0 μmole dithiothreitol; enzyme; 4.0 μmoles 2-ketoisovalerate; and deionized water to 1.0 ml volume. The reaction was started by the addition of the substrate and the change in absorbance at 340 nm was recorded using a Beckman-Gilford spectrophotometer equipped with thermospacers containing circulating water at 30° C. Pyruvate dehydrogenase and 2-ketoglutarate dehydrogenase were assayed using the same reaction composition except for the substitution of 4.0 μmoles of pyruvate or 2-ketoglutarate, respectively. Protein concentrations were determined by the Warburg and Christian method (12) and all specific activities were expressed as μmoles NADH/min/mg protein.

Enzyme Purification. An 8.2 gm sample of cells was thawed and resuspended in 20 ml of cold 0.10 M imidazole-HCl buffer, pH 6.5, containing 1 mM $MgCl_2$, using a glass homogenizer. Cells were broken in a French Pressure Cell Press (American Instrument Co., Inc.) by a single passage through a pre-cooled (4° C) pressure cell. The extract was centrifuged at 21,000 x g for 20 minutes at 0° C. The supernatant fraction was pipetted out of the centrifuge tube and centrifuged at 90,000 x g for 90 minutes at 0° C using a 60-Ti rotor. The supernatant fraction was carefully pipetted out of the centrifuge tube and put into a small beaker that had been pre-cooled to 4° C. Sufficient cold 1% acetic acid was added with stirring to lower the pH to 6.15. 2% Protamine sulfate, pH 6.1, was added with stirring to yield a final concentration of 0.15%. After addition was complete, stirring at 4° C was continued for 15 minutes. This solution was centrifuged at 21,000 x g for 15 minutes at 0° C. The supernatant fraction was decanted and centrifuged at 176,000 x g for 2.5 hours at 0° C using the 60-Ti rotor. The supernatant fraction was decanted and the sediment resuspended in 1 ml of 0.10 M imidazole-HCl buffer. After all assays were completed, the 176,000 x g sediment was diluted with the same buffer to give a protein concentration of 5 mg/ml. Cold 1% acetic acid was added with stirring at 4° C to lower the pH to 5.7. Stirring was continued for 15 minutes, and the sample was centrifuged at 21,000 x g for 15 minutes at 0° C. The supernatant fraction was removed and the pH was lowered to 5.15 using cold 1% acetic acid. After 15 minutes of incubation, the sample was centrifuged at 21,000 x g for 15 minutes at 0° C. The precipitate contained most of the enzyme activity, and was dissolved in 0.4 ml of the 0.10 M imidizole-HCl buffer used previously. The summary of this purification procedure is shown in Table I.

Amino Acid Analysis. Dr. Robert Delaney, University of Oklahoma Health Sciences Center, Department of Biochemistry and Molecular Biology, determined the amino acid content of the heat-treated fraction, using a Biochrom Amino Acid Analyzer (Bio-Cal) equipped with a single 3 mm column containing Durrum DC-6A resin. Analysis was performed with a two buffer system with quantitation by ninhydrin.

RESULTS

The enzyme (Table I) is most active with 2-ketoisovalerate, 2-ketoisocaproate and 2-keto-3-methylvalerate and these three activities are enriched in the same ratio during the purification procedure. The order of activity with the branched chain ketoacids as substrates is 2-ketoisovalerate> 2-keto-3-methylvalerate$\geq$2-ketoisocaproate which is similar to that obtained by Danner et al., (13) with a rat liver enzyme preparation. The preparation illustrated in Table I contained 4.3% of the starting pyruvate dehydrogenase units for a specific activity of 0.11 μmoles per minute per mg protein and 0.6% of the 2-ketoglutarate dehydrogenase units for a specific activity of 0.034 μmoles per minute per mg protein. The purified enzyme also contains dihydrolipoyl dehydrogenase with a specific activity of between 0.4 to 0.6 μmoles per minute per mg protein. The enzyme

Table I **PURIFICATION OF BRANCHED CHAIN KETOACID DEHYDROGENASE FROM P. PUTIDA**

	Fraction	Protein mg	Units	Specific Activity
1.	90,000 x g Supernatant	355	6.4	0.018
2.	Supernatant from protamine sulfate	293	3.5	0.012
3.	176,000 x g pellet	93		
	a. Without heat-treated supernant		2.7	0.029
	b. With heat-treated supernant		3.8	0.041
4.	pH 5.7 Supernatant	40	2.3	0.057*
5.	pH 5.15 Precipitate	6.4	1.6	0.251*

*Assayed with 0.2 ml heat-treated supernatant fraction

has a molecular weight in excess of two million, judging from its elution position from Sepharose 4B. The requirement for a heat-soluble factor became apparent during the assay of the pellet which was formed after centrifugation at 176,000 x g. When the soluble fraction from this step was heated at 65° C for ten minutes and added to fraction 3, there was consistent stimulation of enzyme activity (Table I, Fig. 1A). All subsequent fractions were then assayed in the presence of the heat-treated fraction.

The identity of the heat-stable factor was established as follows: Since the heat treated fraction contained dihydrolipoyl dehydrogenase, the possibility was considered that this enzyme was the unknown factor. However, when the active fraction was heated to 100°C, dihydrolipoyl dehydrogenase was completely inactivated but the soluble fraction still stimulated branched chain ketoacid dehydrogenase. Furthermore, the addition of highly purified dihydrolipoyl dehydrogenase from P. putida did not affect activity of branched chain ketoacid dehydrogenase. The factor passed through an Amicon UM2 filter indicating that its molecular weight was less than 1,000. Several potential effectors were tried and ruled out including AMP, ADP, ATP, FAD and dihydrolipoamide. However, all three branched chain amino acids stimulated enzyme activity and the stimulation of branched chain ketoacid dehydrogenase by L-valine is shown in Fig. 1B. If the stimulation of branched chain ketoacid dehydrogenase by the heat treated fraction was due entirely to valine, we estimated that the concentration of valine in the Amicon filtrate would be approximately 0.5×10^{-3} M. The concentrations of amino acids in the filtrate were: glycine, 0.21×10^{-3} M; alanine, 0.23×10^{-3} M; valine, 0.51×10^{-3} M; isoleucine, trace; and leucine, 0.02×10^{-3} M. Therefore, all of the stimulation of the filtrate could be explained by the presence of valine.

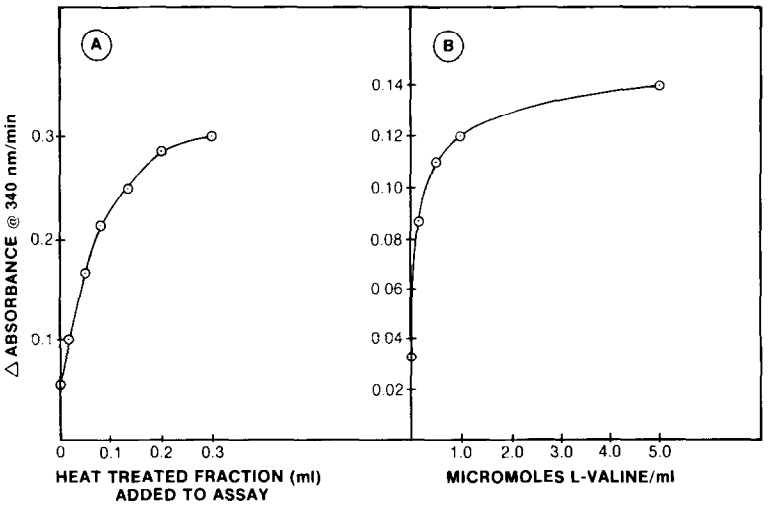

Fig. 1. Stimulation of the standard assay by the heat-treated fraction is shown in Fig. 1A. The amount of enzyme was 0.34 mg protein at a specific activity of 0.135 μmoles NADH/min/mg protein. Fig. 1B shows the stimulation of the standard assay by L-valine. There was 0.12 mg protein in these assays at a specific activity of 0.188 μmoles NADH/min/mg protein.

Stimulation by valine was specific for branched chain ketoacid oxidation since valine had no effect on either pyruvate or 2-ketoglutarate dehydrogenases. However, the heat-treated fraction stimulated 2-ketoglutarate oxidation which means that there must be a separate effector in the heat-treated fraction for 2-ketoglutarate dehydrogenase. Another effect of valine is to stabilize the enzyme. Enzyme prepared as in Table I lost 70% of its activity in 48 hours whereas enzyme prepared in the presence of 5×10^{-3} M valine retained all of its activity for one week, the longest we have kept an enzyme preparation.

The cofactor requirements for branched chain ketoacid dehydrogenase are shown in Table II. The enzyme has an absolute requirement for coenzyme A, branched chain ketoacid, which is used to start the reaction, and NAD. NADP cannot substitute for NAD in the assay. The enzyme is partially resolved for thiamine pyrophosphate since omission of this cofactor reduced the rate of NADH formation by one-half.

It is apparent from the data in Table III that the branched chain amino acids affect the oxidation of the branched chain keto acids in the same relative order, i.e. valine > isoleucine > leucine. The rate of NADH formation is most rapid with 2-keto-isovalerate and about the same with 2-keto-3-methylvalerate and 2-ketoisocaproate. When all three branched chain amino acids are present at 5×10^{-3}M, the rate of NADH formation is stimulated approximately 15% over the rate with valine alone at 5×10^{-3}M, which means that there is no cooperative effect of the branched chain amino acids.

Table II **COFACTOR REQUIREMENTS FOR BRANCHED CHAIN KETOACID DEHYDROGENASE FROM P. PUTIDA**

Assay Components	Nanomoles NADH formed/min	% Relative Activity
1. Complete	41	100
2. - Thiamine pyrophosphate	20	48
3. - L-valine	16	38
4. - Thiamine pyrophosphate, L-valine	7.0	17
5. - CoA	1.4	3.5
6. - CoA, dithiothreitol	0.27	0.66
7. Substitute 5 µmoles D-valine for L-valine	26	63

The enzyme was 0.55 mg protein, specific activity 0.075 µmoles NADH/min/mg protein.

DISCUSSION

To our knowledge, this is the first time that an amino acid has been demonstrated to be an activator of an enzyme in its own catabolic pathway. This observation was first made in our laboratory by V. P. Marshall (14) but could not be confirmed until the enzyme was partially purified. Branched chain ketoacid dehydrogenase of P. putida is the first enzyme which is common to the metabolism of D- and L-valine (1), therefore, it is in a sensitive position to control flow of metabolites through the pathway. We estimate that the intracellular concentration of valine in P. putida grown on valine is 10-20 mM, or ten times that needed for maximum activation of the enzyme. Activation of branched chain ketoacid dehydrogenase may be part of a scheme for regulation of catabolic enzymes for branched chain amino acids, but which does not allow their induction by amino acids of the cell's pool. The fact that branched chain ketoacids and not the amino acids are inducers of branched chain ketoacid dehydrogenase fits this interpretation (2). An interesting feature of the data in Table III is that the branched chain amino acids always stimulate branched chain ketoacid oxidation in the same order; valine > isoleucine > leucine which suggests that the subunit affected is common for all three branched chain ketoacids. The affected subunit must be either the decarboxylase or transacetylase since dihydrolipoyl dehydrogenase catalyzes the same reaction for all ketoacid dehydrogenases.

Table III **STIMULATION OF BRANCHED CHAIN KETOACID DEHYDROGENASE BY BRANCHED CHAIN AMINO ACIDS**

Additions to Standard Assay	Nanomoles NADH/min with		
	2-Ketoiso-valerate	2-Keto-3 methylvalerate	2-Ketoiso-caproate
None	16.4	10.7	9.4
1 µmole L-Valine	46.3	24.0	28.3
1 µmole L-Isoleucine	27.0	18.2	16.2
1 µmole L-Leucine	19.5	14.3	12.4

The enzyme was 0.55 mg protein, specific activity 0.075 µmoles NADH/min/mg protein

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