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D- and L-Isoleucine Metabolism and Regulation of Their Pathways in *Pseudomonas putida*

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Pseudomonas putida oxidized isoleucine to acetyl-coenzyme A (CoA) and propionyl-CoA by a pathway which involved deamination of D-isoleucine by oxidation and L-isoleucine by transamination, oxidative decarboxylation, and beta oxidation at the ethyl side chain. At least three separate inductive events were required to form all of the enzymes of the pathway: D-amino acid dehydrogenase was induced during growth in the presence of D-isoleucine; branched-chain keto dehydrogenase was induced during growth on 2-keto-3-methylvalerate and enzymes specific for isoleucine metabolism; tiglyl-CoA hydrase and 2-methyl-3-hydroxybutyryl-CoA dehydrogenase were induced by growth on isoleucine, 2-keto-3-methylvalerate, 2-methylbutyrate, or tiglate. Tiglyl-CoA hydrase and 2-methyl-3-hydroxybutyryl-CoA dehydrogenase were purified simultaneously by several enzyme concentration procedures, but were separated by isoelectric focusing. Isoelectric points, pH optima, substrate specificity, and requirements for enzyme action were determined for both enzymes. Evidence was obtained that the dehydrogenase catalyzed the oxidation of 2-methyl-3-hydroxybutyryl-CoA to 2-methylacetoacetyl-CoA. 2-Methyl-3-hydroxybutyryl-CoA dehydrogenase catalyzed the oxidation of 3-hydroxybutyryl-CoA, but L-3-hydroxyacyl-CoA dehydrogenase from pig heart did not catalyze the oxidation of 2-methyl-3-hydroxybutyryl-CoA; therefore, they appeared to be different dehydrogenases. Furthermore, growth on tiglate resulted in the induction of tiglyl-CoA hydrase and 2-methyl-3-hydroxybutyryl-CoA dehydrogenase, but these two enzymes were not induced during growth on crotonate or 3-hydroxybutyrate.

A proposal for the metabolism of isoleucine in *Pseudomonas putida*, based on studies with mammalian tissues by Coon and his associates (6, 7) and work with bacteria in our laboratory, is shown in Fig. 1. The initial reactions involve deamination of isoleucine to produce the corresponding keto acid (reactions I.A. and I.B.), oxidative decarboxylation (reaction II), two oxidative reactions (reactions III and V), and finally cleavage to acetyl-coenzyme A (CoA) and propionyl-CoA (reaction VI). Reactions I.A., I.B., and II are referred to as the common pathway (14, 16) since they occur in the metabolism of all three branched-chain amino acids. Branched-chain amino acid transaminase is responsible for deamination of L-branched-chain amino acids (I.A.), D-amino acid dehydrogenase is responsible for deamination of branched-chain D-amino acids (I.B.), and branched-chain keto acid dehydrogenase is responsible for oxidation of branched-chain keto acids (II).

A number of reactions in Fig. 1 have been demonstrated in *Pseudomonas* and other species of bacteria. Marshall and Sokatch (13) prepared a particulate enzyme from *P. aeruginosa*, which was capable of oxidizing several D-amino acids, including D-isoleucine and D-alloisoleucine. This enzyme was inactive with L-amino acids. Norton and Sokatch (21) purified the branched-chain amino acid transaminase (EC 2.6.1.6) from *P. aeruginosa*, which catalyzed transamination of all three branched-chain L-amino acids, as well as L-methionine, L-phenylalanine, and L-norvaline. Deamination of L-isoleucine and D-alloisoleucine would produce L-2-keto-3-methylvaleric acid (Fig. 1). Deamination of D-isoleucine would produce its corresponding D-keto acid, which would be the mirror image of the keto acid shown in Fig. 1.

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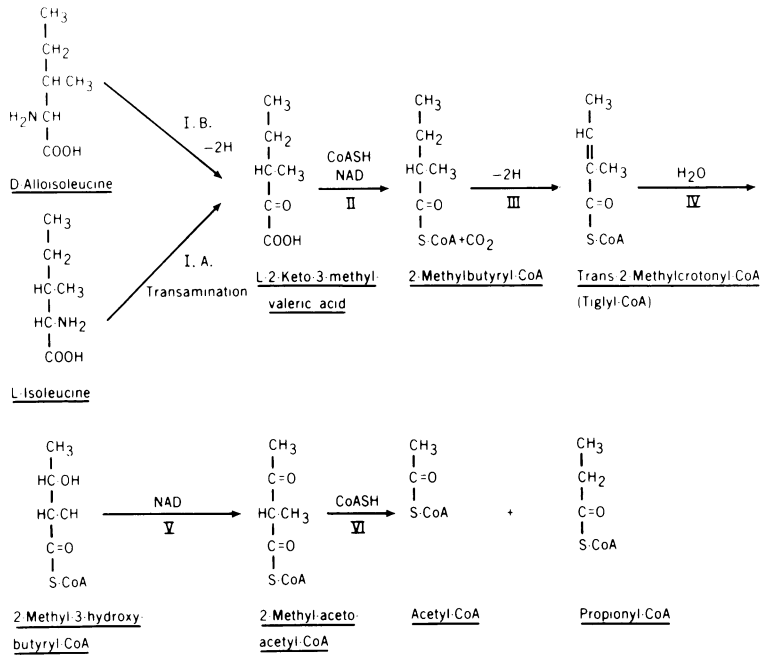

FIG. 1. *Proposed pathway for catabolism of isoleucine in Pseudomonas putida. The reactions in this scheme are catalyzed by: I.A., branched-chain amino acid transaminase; I.B., D-amino acid dehydrogenase; II, branched-chain keto acid dehydrogenase; III, acyl-CoA dehydrogenase; IV, tiglyl-CoA hydrase; V, 2-, ethyl-3-hydroxybutyryl-CoA dehydrogenase; VI, 2-methylacetoacetyl-CoA thiolase.*

Several years ago, Meister (18) showed that *Streptococcus faecalis* and a mutant of *Escherichia coli* which required L-isoleucine for growth were also able to use L- but not D-2-keto-3-methylvaleric acid. *Lactobacillus arabinosus*, however, was able to use L-isoleucine, L-alloisoleucine, and their corresponding keto acids (L-2-keto-3-methylvalerate [Fig. 1] and D-2-keto-3-methylvalerate, respectively) for growth. Reaction II is catalyzed by a branched-chain keto acid dehydrogenase that has been purified from *Bacillus subtilis* (19) and from bovine liver (5). Bowden and Connelly (3) established that there were two separate dehydrogenases in cattle liver, one being responsible for the oxidation of 2-ketoisocaproic and 2-keto-3-methylvaleric acids and the other being responsible for oxidation of 2-ketoisovaleric acid. The latter enzyme activity was also detected in *P. putida* by Marshall (V. P. Marshall, Ph.D. thesis, Univ. of Oklahoma Health Sciences Center, Oklahoma City, 1970). Martin et al. (16) found that a mutant of *P. putida* unable to grow with any of the branched-chain amino acids as sole sources of carbon has lost the ability to oxidize the keto acids derived from valine, leucine, and isoleucine. Very little is known about reaction III catalyzed by branched-chain acyl-CoA dehydrogenase, although a similar activity was reported by Marshall and Sokatch in their study of valine catabolism in *P. putida* (14). Several years ago, Robinson et al. (23) reported that purified crotonase (EC 4.2.17) catalyzed the hydration of tiglyl-CoA to produce 2-methyl-3-hydroxybutyryl-CoA (reaction IV). To our knowledge, there are no reports in the literature concerning reactions V and VI, which are catalyzed by enzymes that we have designated as 2-methyl-3-hydroxybutyryl-CoA dehydrogenase and 2-methylacetoacetyl-CoA thiolase.

The purpose of the present study was to determine the pathway of isoleucine metabolism in *P. putida* and its regulation, and to report on the properties of two enzymes which have not been studied previously: tiglyl-CoA hydrase and 2-methyl-3-hydroxybutyryl-CoA dehydrogenase.

MATERIALS AND METHODS

Organism and culture conditions. *P. putida* PpG2(ATCC 23287) was obtained from I.C. Gunsalus at the University of Illinois. The organism was grown in the basal medium of Jacobson et al. (10), with carbon sources added at the indicated concentrations.

Stock cultures of *P. putida* maintained on tryptose-phosphate agar were inoculated into 50 ml of Jacob-

son medium with 0.3% L-glutamate as the carbon source, and shaken for 8 to 10 h at 30 C. This culture served as the inoculum for other media at the ratio of 1 ml of glutamate culture per 100 ml of medium. Aeration was provided by shaking in a New Brunswick gyratory water bath-incubator (model G76) at 30 C. Growth rates and cell yields were determined by using 250-ml side-arm flasks containing 50 ml of method of Stadtman (26), which was based on the Bausch and Lomb Spectronic 20, and the dry weight was determined by use of a standard curve which equated optical density to dry weight.

Synthesis of substrates and standards. 2-Methyl-3-hydroxybutyrate was synthesized by the method of Blaise and Hermann as modified by Robinson and Coon (24). CoA derivatives were synthesized by the method Stadtman (26), which was based on the procedure of Simon and Shemin (25). CoA derivatives were lyophilized to remove ethyl ether which caused a non-enzymatic reduction of nicotinamide adenine dinucleotide (NAD) in the presence of *P. putida* cell-free extract. The concentration of CoA derivatives was determined by the method of Lipmann and Tuttle (12).

2,4-Dinitrophenylhydrazone standards were made by adding the appropriate ketone or aldehyde directly to 15 mM 2,4-dinitrophenylhydrazine solution in 2 N HCl. When precipitation was complete, the hydrazones were filtered, selectively extracted with ethyl acetate, and dried under vacuum at 45 C. Ethyl acetate was used to recrystallize the hydrazones. All other reagents were commercial preparations.

Enzyme preparation. Cells used for the preparation of enzyme extracts were harvested by centrifugation in late exponential phase, washed once with cold 50 mM potassium phosphate buffer (pH 7.5), and resuspended in the same buffer. Cell-free extract was prepared by sonic oscillation with a Branson model 140 oscillator for 90 to 120 s. Cellular debris was removed by centrifugation, and the supernatant fluid was used as the source of enzyme. Protein concentration was determined by the method of Warburg and Christian (30).

Assays of enzymatic activity. All enzyme activities were assayed spectrophotometrically in a Beckman model DU spectrophotometer equipped with a Gilford model 2000 multiple absorbance recorder. The spectrophotometer was equipped with thermospacers through which water at 30 C was circulated. All specific activities are expressed as nanomoles of product utilized per minute per milligram of protein.

D-Amino acid dehydrogenase was assayed by the method of Norton et al. (20). Branched-chain amino acid transaminase (EC 2.6.1.6) was measured by the assay of Taylor and Jenkins (29). Branched-chain keto acid dehydrogenase was assayed by the method of Marshall and Sokatch (14). 3-Hydroxy-3-methylglutaryl-CoA lyase (EC 4.1.3.4) was measured spectrophotometrically by the method of Stegink and Coon (27).

The assay for 2-methyl-2-hydroxybutyryl-CoA dehydrogenase was developed during this investigation. Enzyme activity was followed spectrophotometrically by measuring the reduction of NAD at 340 nm. The standard assay contained: glycine buffer (pH 9.5), 100 μmol, NAD, 5 μmol; 2-methyl-3-hydroxybutyryl-CoA, 0.40 μmol; enzyme; and water to 1 ml. The control cuvette contained all reagents except substrate. The reaction was initiated by the addition of substrate. One unit of enzyme activity was defined as 1 μmol of reduced NAD (NADH) produced per min.

The assay to measure tiglyl-CoA hydrase was also developed during the course of this study. The assay was based on the oxidation of the product of tiglyl-CoA hydrase activity, 2-methyl-3-hydroxybutyryl-CoA, by 2-methyl-3-hydroxybutyryl-CoA dehydrogenase. Early studies depended on the amount of dehydrogenase present in the enzyme extract, but when the purified dehydrogenase was available a measured excess amount of dehydrogenase was used in the assay. The standard assay contained: glycine buffer (pH 9.5), 100 μmol; NAD, 5 μmol; tiglyl-CoA, 0.40 μmol; 2-methyl-3-hydroxybutyryl-CoA dehydrogenase free of tiglyl-CoA hydrase, 0.20 U; and water to 1 ml. The reaction was initiated by the addition of tiglyl-CoA. One unit of enzyme activity was defined as 1 μmol of NADH produced per min.

The assay for 3-hydroxybutyryl-CoA dehydrogenase was identical to that for 2-methyl-3-hydroxybutyryl-CoA dehydrogenase, except that the substrate was 0.4 μmol of 3-hydroxybutyryl-CoA.

The assay for crotonase was similar to that for tiglyl-CoA hydrase, except that the substrate was crotonyl-CoA and tris(hydroxymethyl)aminomethane buffer (pH 9.0) was used instead of glycine buffer. This was possible because 2-methyl-3-hydroxybutyryl-CoA dehydrogenase was active with 3-hydroxybutyryl-CoA.

Purification of 2-methyl-3-hydroxybutyryl-CoA dehydrogenase and tiglyl-CoA hydrase. Cell-free extracts of *P. putida* grown with 0.3% DL-isoleucine as the energy source were prepared from 30 g (wet weight) of frozen cells suspended in 30 ml of 50 mM potassium phosphate buffer (pH 7.5). The cell suspension was treated with sonic vibration in 10-ml volumes for 150 s. Cellular debris was removed from the recombined fractions by centrifugation at $10^4 \times g$ for 30 min.

The cell-free extract was treated with ammonium sulfate, and the precipitate which formed between 30 and 60% ammonium sulfate was collected by centrifugation at $40,000 \times g$ for 40 min. The concentration of ammonium sulfate was calculated by the method of Brenner-Holzach and Staehelin (4). The white precipitate was dissolved in 5 ml of 50 mM potassium phosphate buffer (pH 7.5) and dialyzed overnight against 1 liter of the same buffer.

The dialysate was placed on a diethylaminoethyl (DEAE)-cellulose column (25 by 400 mm) and eluted with the same buffer. Fractions of 9 ml were collected, and both enzymes were detected just beyond the holdup volume in fractions 14 to 18, which were then pooled. The hydrase and dehydrogenase activities were separated by isoelectric focusing (11) using a pH gradient from 3 to 10. The entire column (440 ml) was collected in 200-drop fractions after 36 h of focusing at 400 V.

Disc-gel electrophoresis was performed by the

method of Davis (8) and Ornstein (22). Protein was fixed and stained by a solution containing 10% trichloroacetic acid and 0.12% Coomassie brilliant blue.

Hydroxymethylglutaryl-CoA lyase. The partially purified 3-hydroxy-3-methylglutaryl-CoA lyase (EC 4.1.3.4) used in studies of the substrate specificity of tiglyl-CoA hydrase (see Table 5) was prepared by sonic oscillation of 15 g (wet weight) of *P. putida* grown in a medium with 0.3% DL-leucine. The extract was treated with solid ammonium sulfate, and the fraction which precipitated between 45 and 65% saturation was dissolved in 5 ml of 5 mM phosphate buffer (pH 7.5) and dialyzed overnight against the same buffer. The subsequent volume (11.7 ml) was applied to a DEAE-cellulose column (25 by 400 mm) and eluted with 400 ml of phosphate buffer (pH 7.5) from a reservoir designed to produce a linear gradient from 5 to 40 mM. Hydroxymethylglutaryl-CoA lyase was eluted with a peak of activity at approximately 200 ml and had a specific activity of 3 μmol per min per mg of protein by the assay system of Stegink and Coon (27).

Identification of the product of 2-methyl-3-hydroxybutyryl-CoA dehydrogenase activity. The enzyme preparation used to determine the end product of 2-methyl-3-hydroxybutyryl-CoA dehydrogenase had been enriched by DEAE-cellulose column chromatography and had a specific activity of 1.5 μmol of NADH per min per mg of protein. The complete reaction mixture contained: glycine buffer (pH 9.5), 300 μmol; NAD, 25 μmol; protein, 0.22 mg; 2-methyl-3-hydroxybutyryl-CoA, 7 μmol; and water to 5 ml. One control reaction contained boiled enzyme; a second control lacked enzyme and substrate but contained 2 μmol of redistilled methyl ethyl ketone. Progress of the reaction was monitored spectrophotometrically. After NAD reduction had ceased, 0.2 ml of 3.5 N NaOH was added to each tube to hydrolyze the CoA derivatives. After 1 h, the solutions were titrated to pH 3.5 to 4.0 with 1.0 M H_2SO_4 and kept overnight at room temperature to effect the decarboxylation of 2-methylacetoacetate to methyl ethyl ketone. 2-Methyl-3-hydroxybutyrate is stable under these conditions. Each tube was extracted three times with an equal volume of ethyl ether, and then 2 ml of 15 mM 2,4-dinitrophenylhydrazine reagent in 2 N HCl was added to the combined ether extracts. After 1 h, the solutions were extracted successively with 10 ml of 1.0 M H_2SO_4, 10 ml of 0.1 N Na_2CO_3-0.01 N $NaHCO_3$, and 10 ml of water. The organic phase was taken to dryness under nitrogen, dissolved in 0.05 ml of ethyl acetate, spotted on thin-layer plates of activated aluminum oxide G, and developed in *n*-hexane-benzene (1:1). The spots were scraped off the plate, eluted with ethyl acetate, and rechromatographed to produce spots which did not streak. R_{for} was calculated by the method of Denti and Luboz (9; see Table 8).

RESULTS

Generation times. Generation times for growth with DL-, D-, or L-isoleucine were of the order of 5 h (Table 1), although the total cell yield with DL-isoleucine was less than with the other isomers of isoleucine. The lowered cell yield with DL-isoleucine, a synthetic substrate, may be due to the presence of L-alloisoleucine, which Meister (18) noted could not be used by bacteria which required isoleucine for growth. Generation times for growth with valine were 8 to 11 h, and there appeared to be very little difference in the results obtained with the isomers. However, the generation time with L-leucine as the carbon and energy source was 20 h compared to 3.7 h with D-leucine, a result that suggested that L-leucine was either inhibitory to growth or was metabolized slowly. This result is discussed further in the accompanying paper (17). The observation that isoleucine overcomes valine toxicity in *P. putida* has already been reported (10, 14).

TABLE 1. *Generation times and cell yields of P. putida grown on isomers of branched-chain amino acids*[a]

Substrate	Generation time (h)
DL-Isoleucine (four isomers)	4.7
D-Isoleucine (with allo)	5.1
L-Isoleucine (allo-free)	5.5
DL-Valine	10.4
D-Valine	11.4
L-Valine	8.0
DL-Leucine	3.3
D-Leucine	3.7
L-Leucine	20.4
D-Glucose	1.2
L-Glutamate	1.0

[a] Generation times were determined in media with 0.3% carbon source.

Induction of isoleucine catabolic enzymes. The data in Tables 2 and 3 are in agreement with earlier observations that two enzymes of the common pathway, D-amino acid dehydrogenase and branched-chain keto acid dehydrogenase, were induced by growth with any of the three branched-chain amino acids and that branched-chain keto acid dehydrogenase, but not D-amino acid dehydrogenase, was induced by growth in media with 2-keto-3-methylvalerate (14, 16). Branched-chain amino acid transaminase is a constitutive enzyme (14). High levels of tiglyl-CoA hydrase, 2-methyl-3-hydroxybutyryl-CoA dehydrogenase, and 2-methylacetoacetyl-CoA thiolase were specifically induced by growth on DL-isoleucine, 2-keto-3-methylvalerate, 2-methylbutyrate, and tiglate, all of which are themselves intermediates or their coenzyme A derivatives are intermediates in the isoleucine catabolic pathway.

TABLE 2. *Comparison of isoleucine catabolic enzymes in P. putida grown on different carbon sources*

Enzyme	Sp act (nmol/min/mg of protein) when carbon source for growth (0.3%, wt/vol) was:					
	DL-Isoleucine	DL-Valine	DL-Leucine	Succinate	D-Glucose	L-Glutamate
D-Amino acid dehydrogenase	15.8	6.0	1.8	0	0	0
Branched-chain keto acid dehydrogenase	7.5	3.0	10.4	0	0	0
Tiglyl-CoA hydrase[a]	196	29	5	10.2	3.7	2.7
2-Methyl-3-hydroxybutyryl-CoA dehydrogenase	183	40	22	24	1.2	10.1

[a] Assay for tiglyl-CoA hydrase was limited by the amount of 2-methyl-3-hydroxybutyryl-CoA dehydrogenase in extracts.

TABLE 3. *Comparison of inducible isoleucine catabolic enzymes in P. putida grown on intermediates of pathway*

Enzyme	Sp act (nmol/min/mg of protein) when carbon source for growth (0.3% wt/vol) was:					
	DL-Isoleucine	2-Keto-3-methylvalerate	2-Methyl-butyrate	Tiglate	L-Glutamate	D-Glucose
D-Amino acid dehydrogenase	4.3	0	0	0	0	0
Branched-chain keto acid dehydrogenase	26	10.4	0	0	0	0
Tiglyl-CoA hydrase[a]	239	113	170	221	4	5
2-Methyl-3-hydroxybutyryl-CoA dehydrogenase	132	149	147	334	28	11

[a] Assay for tiglyl-CoA hydrase was limited by the amount of 2-methyl-3-hydroxybutyryl-CoA dehydrogenase in extracts.

When the extracts were supplemented with excess 2-methyl-3-hydroxybutyryl-CoA dehydrogenase, higher levels of tiglyl-CoA hydrase were detected (Table 4) than in earlier studies when rates were limited by the amount of 2-methyl-3-hydroxybutyryl-CoA dehydrogenase present in the extract (Tables 2 and 3). However, the level of hydrase observed with *P. putida* grown with 2-keto-3-methylvalerate was about equal to that obtained with DL-valine. The reason for the low level of hydrase after growth with 2-keto-3-methylvalerate is unknown, but the apparent induction by valine is not surprising in view of the steric similarities of valine and isoleucine. Significant levels of 2-methyl-3-hydroxybutyryl-CoA dehydrogenase were obtained only when tiglate, DL-isoleucine, or 2-keto-3-methylvalerate was used as the carbon and energy source.

Purification of tiglyl-CoA hydrase. During our early studies of the purification of enzymes of isoleucine catabolism, we found that 2-methyl-3-hydroxybutyryl-CoA dehydrogenase and tiglyl-CoA hydrase purified together, but that they could be separated by isoelectric focusing (Fig. 2). Tiglyl-CoA hydrase obtained by this procedure had a specific activity of about 30 μmol of NADH per min per mg of protein when assayed with the coupled reaction using excess 2-methyl-3-hydroxybutyryl-CoA dehydro-

TABLE 4. *Induction of tiglyl-CoA hydrase and 2-methyl-3-hydroxybutyryl-CoA in P. putida*

Growth substrate (0.3%, wt/vol)	Sp act (nmol/min/mg of protein)	
	Tiglyl-CoA hydrase[a]	2-Methyl-3-hydroxy-butyryl-CoA dehydro-genase
Tiglate	3,420	208
DL-Isoleucine	2,820	161
2-Keto-3-methylvalerate	630	106
DL-Valine	645	30
DL-Leucine	368	12
3-Hydroxybutyrate	146	0
Crotonate	154	8
Acetate	157	7
Glutamate	120	8

[a] 2-Methyl-3-hydroxybutyryl-CoA dehydrogenase (0.21 U) was added to assay.

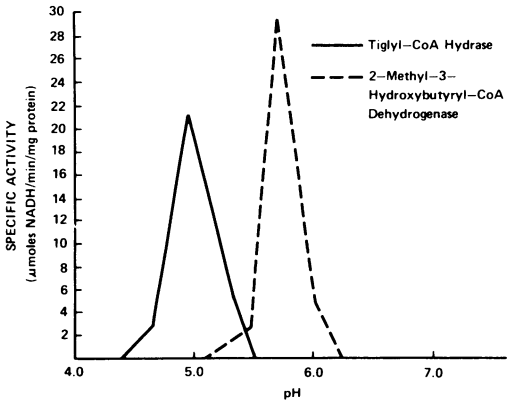

FIG. 2. *An LKB analytical isoelectric focusing column was used with broad-spectrum ampholine (pH 3 to 10). The initial voltage was 400 V and current was 30 mA. The current stabilized after 36 h at 4 C and electrophoresis was terminated. Fractions of 200 drops were collected and the pH was determined for each fraction. The isoelectric point for each enzyme was determined by measuring the pH of the pooled fractions containing enzyme of the highest specific activity.*

genase. The enzyme obtained by isoelectric focusing was not completely pure, but was composed of two major proteins and several minor proteins as determined by the disc electrophoresis. The isoelectric point determined by this procedure was 5.1, and the optimal pH for enzyme activity was 9.9, which is similar to that of crotonase from beef liver (28). Tiglyl-CoA hydrase was active with both crotonyl-CoA and tiglyl-CoA when the coupled assay with 2-methyl-3-hydroxybutyryl-CoA dehydrogenase was used (Table 5). It is significant that no activity was obtained with 3-methylglutaconyl-CoA, an intermediate in leucine metabolism, with hydroxymethylglutaryl-CoA lyase as coupling enzyme. We do not have enough data to determine whether the tiglyl-CoA hydrase purified in this study is different from the classical crotonase, but the facts that it co-purifies to a certain extent with 2-methyl-3-hydroxybutyryl-CoA dehydrogenase, that it appears to be induced by growth on substrates related to isoleucine catabolism (Table 4), and that it appears to be inactive on 3-methylglutaconyl-CoA indicate that further study of this enzyme is warranted.

2-Methyl-3-hydroxybutyryl-CoA dehydrogenase. The data for a typical purification of 2-methyl-3-hydroxybutyryl-CoA dehydrogenase from *P. putida* are shown in Table 6. The enzyme obtained by this procedure had only one major band after disc-gel electrophoresis, but occasionally minor bands were detected. Estimations of molecular weight by sucrose gradients (15) and Sephadex G-200 gel filtration (1) gave values close to 130,000. The purified enzyme catalyzed the oxidation of 2-methyl-3-hydroxybutyryl-CoA with NAD as the electron acceptor. NADP and 2-methyl-3-hydroxybutyric acid plus free coenzyme A were unable to substitute for NAD and 2-methyl-3-hydroxybutyryl-CoA. The pH optimum for the reaction was approximately 9.5 (Table 7).

Evidence that the product of the reaction was 2-methylacetoacetyl-CoA was obtained by identifying the 2,4-dinitrophenylhydrazone derivative of the product of the reaction. 2-Methylacetoacetyl-CoA would yield the 2,4-dinitrophenylhydrazone of methyl ethyl ketone by the method used, and this derivative was

TABLE 5. *Substrate specificity of tiglyl-CoA hydrase*

Substrate	Sp act (μmol/min/mg of protein)
Tiglyl-CoA[a]	7.7
Crotonyl-CoA[a]	11.2
3-Methylglutaconyl-CoA[b]	0

[a] Each assay contained 0.10 U of 2-methyl-3-hydroxybutyryl-CoA dehydrogenase and 4 μg of tiglyl-CoA hydrase (specific activity 32.5 μmol per min per mg of protein). Both enzymes were dialyzed overnight against 50 mM phosphate buffer (pH 7.5) to remove ampholine, which appeared to cause nonenzymatic hydration of crotonyl-CoA.

[b] This reaction mixture contained 0.17 U of partially purified 3-hydroxy-3-methylglutaryl-CoA lyase (EC 4.1.3.4) instead of the dehydrogenase.

TABLE 6. *Purification of 2-methyl-3-hydroxybutyryl-CoA dehydrogenase from P. putida*

Determination	Total protein (mg)	Total U	Sp act (μmol/min/mg of protein)	Purification (fold)	Recovery (%)
Enzyme	2,268	680	0.299	1	100
Precipitate obtained with ammonium sulfate from 35 to 60% saturation	607	457	0.754	2	67
Pool from DEAE-cellulose	12	148	12.4	41	21
Pool from isoelectric focusing	0.76	30.6	40.3	134	4.5

identified by thin-layer chromatography (Table 8). Controls using boiled enzyme and native enzyme without substrate but with added methyl ethyl ketone confirmed that there was no product produced with inactive enzyme and that the hydrazone of methylethyl ketone could be isolated from the enzymatic reaction mixture.

2-Methyl-3-hydroxybutyryl-CoA dehydrogenase was active with 2-methyl-3-hydroxybutyryl-CoA and 3-hydroxybutyryl-CoA (Table 9), and to a very slight extent with 2-hydroxy-3-methylvaleryl-CoA. None of the other substrates tested was active, nor was there any activity with the free acids plus CoA. A study of the enzyme kinetics revealed that the Michaelis constant (K_m) for 2-methyl-3-hydroxybutyryl-CoA was 3.7×10^{-5} M and that the K_m for 3-hydroxybutyryl-CoA was 5×10^{-4} M. Maximal velocities (V_{max}) in this same experiment were 37 nmol of NADH per min for 2-methyl-3-hydroxybutyryl-CoA and 83 nmol of NADH per min with 3-hydroxybutyryl-CoA. At 10^{-4} M substrate, the enzyme was only half as active with 3-hydroxybutyryl-CoA as with 2-methyl-3-hydroxybutyryl-CoA because of the difference in K_m values. It should also be noted that both substrates were synthetic, and we would expect that there would be four isomers of 2-methyl-3-hydroxybutyrate and two isomers of 3-hydroxybutyrate present in our substrates.

TABLE 7. *Components necessary for activity of 2-methyl-3-hydroxybutyryl-CoA dehydrogenase*

Test system	Sp act (μmol/min/mg of protein)
Complete	7.0
Substitute NADP for NAD	0
Boiled enzyme	0
Omit 2-methyl-3-hydroxybutyryl-CoA	0
Substitute 2-methyl-3-hydroxybutyrate plus free coenzyme A for 2-methyl-3-hydroxybutyryl-CoA	0

TABLE 8. *Thin-layer chromatography of 2,4-dinitrophenylhydrazone of product of 2-methyl-3-hydroxybutyryl-CoA dehydrogenase action*

2,4-Dinitrophenylhydrazone from:	R_{for}[a]
Reaction with native enzyme	1.77
Methyl ethyl ketone standard	1.72[b]
Reaction with no substrate + methyl ethyl ketone	1.66
Acetone standard	1.22
Formaldehyde standard	1.00
Reaction with boiled enzyme	0

[a] R_{for} = R_f of compound hydrazone/R_f of formaldehyde hydrazone.

[b] Literature value reported by Denti et al. (9) was 1.64.

TABLE 9. *Substrate specificity of purified 2-methyl-3-hydroxybutyryl-CoA dehydrogenase*

Substrate	Sp act (μmol/min/mg of protein)
2-Methyl-3-hydroxybutyryl-CoA	16.2
3-Hydroxybutyryl-CoA	21.7
2-Hydroxy-3-methylvaleryl-CoA	0.4
3-Hydroxyisobutyryl-CoA	0
2-Ethyl-2-hydroxybutyryl-CoA	0
2-Methyl-2-hydroxybutyryl-CoA	0
2-Hydroxyisobutyryl-CoA	0
3-Hydroxy-3-methylglutaryl-CoA	0

2-Methyl-3-hydroxybutyryl-CoA dehydrogenase is apparently different from 3-hydroxyacyl-CoA dehydrogenase. A commercial preparation of pig heart L-3-hydroxyacyl-CoA:NAD oxidoreductase (EC 1.1.1.35) was inactive with 2-methyl-3-hydroxybutyryl-CoA. Furthermore, when commercial L-3-hydroxyacyl-CoA dehydrogenase was allowed to react to completion with DL-3-hydroxybutyryl-CoA, the remaining material was not a substrate for 2-methyl-3-hydroxybutyryl-CoA dehydrogenase and vice versa. These results suggest that the substrate for our enzyme has the L-configuration.

When we found that 2-methyl-3-hydroxybutyryl-CoA dehydrogenase was active with 3-hydroxybutyryl-CoA, we decided to study the enzyme patterns of *P. putida* grown on tiglate, crotonate, and 3-hydroxybutyrate (Table 10). The results obtained in this study were significant, since they showed that growth of *P. putida* on tiglate resulted in high levels of tiglyl-CoA hydrase and 2-methyl-3-hydroxybutyryl-CoA dehydrogenase, as well as 3-hydroxybutyryl-CoA dehydrogenase and crotonase. However, growth on crotonate or 3-hydroxybutyrate resulted in elevated levels only of 3-hydroxybutyryl-CoA dehydrogenase and crotonase. These results suggest that tiglyl-CoA hydrase and 2-methyl-3-hydroxybutyryl-CoA dehydrogenase are different enzymes from crotonase and 3-hydroxybutyryl-CoA dehydrogenase.

DISCUSSION

The purpose of this study was to elucidate the pathway used by *P. putida* for the catabolism of isoleucine and to study the regulation of this

TABLE 10. *Induction of isoleucine and 3-hydroxybutyrate catabolic enzymes in P. putida*

Enzyme	Sp act (nmol/min/mg of protein)[a] when carbon source for growth was:			
	0.3% Tiglate	0.3% Crotonate	0.3% 3-Hydroxybutyrate	0.3% L-glutamate
Tiglyl-CoA hydrase	170	16	18	16
2-Methyl-3-hydroxybutyryl-CoA dehydrogenase	302	26	27	26
3-Hydroxybutyryl-CoA dehydrogenase	429	260	590	55
Crotonase	235	137	80	12

[a] Measurement of the rate of which the enoyl-CoA derivatives were hydrated was limited by the amount of dehydrogenase present in the extract.

pathway. These objectives were achieved, and most of the reactions shown in Fig. 1 were demonstrated. Studies from this laboratory have shown that the general outline for the pathway of the metabolism of all three branched-chain amino acids is composed of a common pathway for the deamination of the D- and L-isomers of the amino acids and oxidative decarboxylation of the keto acids, followed by a specific pathway for the metabolism of each amino acid catalyzed by unique enzymes. Likewise, the regulation of these pathways is similar and is composed of at least three separate inductive events, one for the D-amino acid dehydrogenase, one for the branched-chain keto acid dehydrogenase, and one for induction of the enzymes unique to the metabolism of valine, isoleucine, and leucine. The branched-chain amino acid transaminase, which is responsible for the deamination of L-amino acids, is a constitutive enzyme in *P. putida* (14). The role of this enzyme in the cell must be an interesting one if it functions in both catabolism and biosynthesis of the branched-chain amino acids. It would be interesting to determine how the cell distinguishes between keto acids derived from catabolic pathways, which cause induction of branched-chain keto acid dehydrogenase (16), and keto acids derived from biosynthetic pathways, which do not induce branched-chain keto acid dehydrogenase.

At least one and possibly two new enzymes were characterized during the course of this study, tiglyl-CoA hydrase and 2-methyl-3-hydroxybutyryl-CoA dehydrogenase. The action of tiglyl-CoA hydrase in isoleucine catabolism is an interesting one, since it was found to catalyze the hydration of both tiglyl-CoA and crotonyl-CoA in extracts prepared from cells grown on isoleucine and since tiglyl-CoA hydrase activity was very low in extracts prepared from cells grown on 3-hydroxybutyrate. These facts lead us to believe that tiglyl-CoA hydrase, which was purified in this study, is probably an enzyme unique to isoleucine metabolism, but the point needs further study. It was also interesting that tiglyl-CoA hydrase and 2-methyl-3-hydroxybutyryl-CoA dehydrogenase co-purified during ammonium sulfate fractionation, ion exchange chromatography, and gel filtration.

We are reasonably sure, however, that 2-methyl-3-hydroxybutyryl-CoA dehydrogenase is an enzyme which is unique to isoleucine metabolism and different from 3-hydroxybutyryl-CoA dehydrogenase.

The facts that purified pig heart 3-hydroxybutyryl-CoA dehydrogenase had no activity on 2-methyl-3-hydroxybutyryl-CoA and that 2-methyl-3-hydroxybutyryl-CoA dehydrogenase activity is absent from cells grown on hydroxybutyrate lead us to this conclusion.

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