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Metabolism of β-Methylaspartate by a Pseudomonad

O. K. SEBEK[1] AND H. A. BARKER
Department of Biochemistry, University of California, Berkeley, California 94720

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A bacterium was isolated from soil which utilizes *threo*-β-methyl-L-aspartate, certain other amino acids, and a variety of organic substances as single energy sources. It is, or closely resembles, *Pseudomonas putida* biotype B. The ability of this organism to rapidly decompose such amino acids is dependent on inducible enzyme systems. Dialyzed cell-free extracts of this bacterium metabolize β-methylaspartate only when catalytic amounts of α-ketoglutarate, or pyruvate, and pyridoxal phosphate are also present. The main products formed from β-methylaspartate under these conditions are α-aminobutyrate, carbon dioxide, and α-ketobutyrate. When L-aspartate is substituted for β-methylaspartate in this system, it is converted mainly to alanine and carbon dioxide. β-Methyloxalacetate is decarboxylated, and the resulting α-ketobutyrate is converted enzymatically in the presence of glutamate to α-aminobutyrate which accumulates. The added keto acids are converted, in part, to the corresponding amino acids probably by transamination. The data indicate that β-methylaspartate is converted to α-aminobutyrate, and aspartate to alanine, by a circuitous transamination-β-decarboxylation-transamination sequence rather than by a direct β-decarboxylation.

β-Methylaspartate has been shown to be an intermediate in the fermentation of glutamate by *Clostridium tetanomorphum* (3, 4). In this bacterium, β-methylaspartate is formed from L-glutamate and is subsequently deaminated to mesaconate, which in turn is converted first to L-citramalate and then to pyruvate and acetate. In contrast, little is known about the metabolism of β-methylaspartate in aerobic microorganisms. Some evidence indicates that the glutamate synthesis in *Acetobacter suboxydans* (13) may involve a reversal of the reaction sequence observed in *Clostridium*. Extracts of *Escherichia coli* catalyze a transamination between β-methylaspartate and α-ketoglutarate to form glutamate and presumably β-methyloxalacetate. The latter compound is rapidly decarboxylated to α-ketobutyrate, a known intermediate in the biosynthesis of isoleucine. Growing cells of *E. coli* incorporate ^{14}C from ^{14}C-methyl-labeled *threo*-β-methyl-DL-aspartate into carbon 5 of isoleucine (1).

This paper reports observations on the decomposition of β-methylaspartate and related compounds by cell-free extracts of a soil pseudomonad.

[1] Research Associate, 1966-1967. On leave of absence from the Department of Microbiology, The Upjohn Company, Kalamazoo, Mich. 49001.

MATERIALS AND METHODS

A motile aerobic bacterium, isolated from soil following enrichment in a liquid medium which contained *threo*-β-methyl-L-aspartate as the sole energy source, was used in this study. It was routinely maintained on slants of Proteose Peptone (0.5%) Agar (Difco). To establish the taxonomic position of this organism, methods and criteria used by Stanier et al. (16) were followed. For experimental purposes, the cells were grown aerobically (200 ml in a 1-liter Erlenmeyer flask) at 30 C in a medium containing 10 to 50 mM β-methylaspartate or other suitable amino acid, 0.1% yeast extract, 0.1% KH_2PO_4, 0.05% $MgSO_4$, 0.5% KCl, and 0.001% $FeSO_4 \cdot 7H_2O$, adjusted with KOH to *p*H 6.4. The medium was aerated vigorously on a rotary shaker at 30 C, and the bacteria were harvested toward the end of the exponential phase of growth. The cells were washed once with water. For manometric experiments, they were resuspended in 50 mM potassium phosphate buffer, *p*H 7.0, and shaken for 18 hr at 30 C to decrease the endogenous respiration. Cell-free extracts were prepared by sonically treating cell suspensions for 8 min in a 10-kc Raytheon sonic oscillator. The centrifuged extracts were used either as such or after dialysis against 50 mM phosphate buffer, *p*H 7.0. Extracts remained active after storage for 2 months at −15 C. Protein was determined by the method of Lowry et al. (12) using bovine serum albumin as standard.

By the method of Barker et al. (6), *threo*-β-methyl-L-aspartic acid was prepared from diammonium mesaconate. The ^{14}C-labeled form of this acid was

prepared from glutamate-UL-^{14}C by a two-step enzymatic conversion. In the first step, ^{14}C-glutamate was converted to ^{14}C-mesaconate in 52% yield by the combined action of glutamate mutase (5) and β-methylaspartase (7). The ^{14}C-labeled mesaconic acid was isolated from the acidified reaction mixture by extraction with ether. The ammonium salt of ^{14}C-mesaconate was then converted to *threo*-β-methyl-L-aspartate with β-methylaspartase isolated from *C. tetanomorphum*. The overall yield was about 10%.

The anhydride of β-methyloxalacetic acid was synthesized (by E. J. Wawszkiewicz) from tertiary butyl propionate and di-tertiary butyl oxalate by the method of Heidelberger and Hurlbert (11). The melting point of the crystalline product was 118 to 119 C, as reported by Abramsky and Shemin (1). Solutions of the sodium salt of methyloxalacetic acid were prepared immediately before use by dissolving the anhydride in the calculated amount of cold 50 mM NaOH.

Paper ionophoresis was carried out by the method of Crestfield and Allen (9) by using either a formic acid (2.5%): acetic acid (7.8%) solution, *p*H 1.85 (2), 50 mM sodium formate buffer, *p*H 3.85, or 30 mM ammonium acetate buffer, *p*H 5.0. The solvents used for paper chromatographic determinations were 1-butyl alcohol-acetic acid-water (40:10:50, w/v, upper phase) or pyridine-isoamyl alcohol-diethylamine-water (50:50:15:35, v/v). Amino acids were detected by means of ninhydrin (0.1% in acetone).

RESULTS

Characteristics, growth requirements, and identification of the organism. The bacterium forms gram-negative, short, stubby rods which occur singly, in pairs, or in short chains. It is motile by means of three polar flagella and elaborates fluorescent pigment on King B diagnostic medium. It grows aerobically at 4, 28, and 37 C but not at 41 C. Its energy-yielding metabolism is respiratory; it is oxidase-positive and does not use NO_3^- as terminal electron acceptor. It does not hydrolyze gelatin or Tween 80. The egg-yolk reaction is negative. It elaborates arginine dihydrolase constitutively. For growth, it can utilize both NH_4^+ and NO_3^- as N sources and, in addition to β-methylaspartate, it uses a variety of organic substances as C sources: L-α-alanine, β-alanine, L-arginine, DL-asparagine, L-aspartate, L-glutamate, L-glutamine, L-histidine, DL-norleucine, L-phenylalanine, L-tryptophan, and L-valine; D-galactose, D-glucose, D-fructose, trehalose, D-ribose, L-arabinose, and D,L-xylose; inositol, mannitol, and glycerol; gluconate and 2-ketogluconate; citrate, succinate, fumarate, malate, citramalate, mesaconate, glutarate, and glycolate; butyrate, isobutyrate, lactate, propionate, acetate and pelargonate; ethyl alcohol, 1-propanol, and 1-butyl alcohol; *p*-hydroxybenzoate, salicylate, phenylacetate, and nicotinate; spermine, acetamide, and triacetin. It grows poorly on kynurenine, glycine, L-leucine, L-proline, DL-serine, L-sorbose, D-mannose, maltose, 1,2-propanediol, sarcosine, and hippurate. No growth was noted on α-aminobutyrate, L-isoleucine, L-lysine, and L-threonine; starch, inulin, sucrose, lactose, rhamnose, D-sorbitol, *m*-erythritol, adonitol, 1,2-ethanediol, 2,3-butanediol, methanol, and geraniol; salicin and testosterone; tryptamine, creatine, benzylamine, naphthalene, and anthranilate. These characteristics permitted us to conclude that our isolate is, or resembles very closely, *Pseudomonas putida* biotype B as defined by Stanier et al. in their extensive taxonomic study of aerobic pseudomonads (16).

Oxidation of amino acids and related organic acids by cell suspensions. In manometric experiments, cells grown in a β-methylaspartate-yeast extract medium readily oxidized β-methylaspartate. In a typical experiment, the rate of O_2 uptake was 0.27 μliter per min per mg (dry weight) of cells at 30 C, or about 6.1 times the endogenous rate. The rates of O_2 uptake with L-aspartate, L-glutamate, or α-ketobutyrate were essentially the same as that with β-methyl-L-aspartate. With L-α-aminobutyrate, the rate was about one-half as large (0.14 μliter of O_2 per min per mg), and with mesaconate or DL-citramalate, the oxygen uptake rate was only slightly above that of the endogenous control.

Cells grown in an aspartate-yeast extract medium oxidized aspartate and glutamate relatively rapidly (0.23 μliter of O_2 per min per mg), and β-methylaspartate or α-ketobutyrate about 40% as fast. The rate of O_2 uptake with β-methylaspartate was constant throughout a 90-min incubation. With α-ketobutyrate as substrate, the rate declined gradually and was slower than with β-methylaspartate after about 60 min.

Cells grown in a glutamate-yeast extract medium oxidized glutamate most rapidly (0.30 μliter of O_2 per min per mg), and β-methylaspartate scarcely at all (0.018 μliter of O_1 per min per mg).

Enzymatic conversion of β-methylaspartate to α-aminobutyrate. A reaction mixture containing 10 mM sodium β-methylaspartate, 10 mM sodium α-ketoglutarate, 0.2 mM pyridoxal phosphate, 50 mM potassium phosphate buffer (pH 7.4), and 4.4 mg of extract protein (from cells grown in a β-methylaspartate-yeast extract medium) in a total volume of 0.50 ml was incubated for 30 min at 37 C. The reaction was stopped by the addition of perchloric acid (4% final concen-

tration). After centrifugation, the supernatant solution was neutralized with KOH and centrifuged. Portions (10 μliters) of the solution (1.0 ml) were used for paper ionophoresis at *p*H 1.85 (38 v per cm, 45 min) and at *p*H 3.85 (38 v per cm, 50 min).

The ionophoretogram at *p*H 3.85 showed three ninhydrin-positive spots. Two anionic spots moved at the same rate as glutamate (4.5 cm) and β-methylaspartate (8.6 cm), whereas the third intense spot moved slightly (1.2 cm) toward the anode indicating a neutral amino acid. In a separate experiment, it was found that the intensity of the latter spot increased markedly with the length of incubation of the reaction mixture. The ionophoretograms of this reaction mixture at *p*H 1.85 showed three ninhydrin-positive spots, all of which moved as cations. They corresponded to β-methylaspartate (5.0 cm), glutamate (6.3 cm), and to an unknown amino acid (8.2 cm) that moved the same as α-aminobutyrate acid. The unknown amino acid in another portion of the reaction mixture was separated from other amino acids by ascending paper chromatography using a butyl alcohol-acetic acid-water solvent. A prominent amino acid acid band corresponding to α-aminobutyrate (R_F 0.25) was eluted from the dried paper with water, evaporated to dryness, and redissolved in 50 μliters of water. On rechromatography on paper, this material appeared as a single spot and had the same mobility as α-aminobutyrate in butyl alcohol-acetic acid-water and in pyridine-isoamyl alcohol-diethylamine-water (R_F 0.27). It also had the same mobility as α-aminobutyrate when examined by paper ionophoresis at *p*H 1.85.

The above evidence indicates that α-aminobutyrate and glutamate are formed from β-methylaspartate and α-ketoglutarate. Paper chromatographic examinations of reaction mixtures of separate experiments showed that the β-methylaspartate decomposition was greatly decreased when α-ketoglutarate was omitted. However, a catalytic amount of α-ketoglutarate (1 mM) was sufficient to permit a complete, though slower breakdown of β-methylaspartate. Omission of pyridoxal phosphate also significantly decreased the rate of β-methylaspartate decomposition.

Pyruvate could replace α-ketoglutarate as a cosubstrate for β-methylaspartate decomposition; the products were then α-aminobutyrate and alanine. When β-methylaspartate was replaced by L-aspartate, in the presence of α-ketoglutarate, the products were alanine and glutamate.

The time course of the enzymatic conversion of β-methylaspartate to α-aminobutyrate and α-ketobutyrate was followed in an experiment with ^{14}C-labeled substrate. Samples (20 μliters) were removed at appropriate intervals and subjected to paper ionophoresis at *p*H 5.9 (36 v per cm, 70 min). Under these conditions, β-methylaspartate and α-ketobutyrate moved 9.0 and 15.2 cm toward the anode, respectively, and α-aminobutyrate moved 2.7 cm toward the cathode.

Utilization of β-methylaspartate was accompanied by a continuous accumulation of α-aminobutyrate (Fig. 1). The yield of α-aminobutyrate after 120 min of incubation was estimated to be 0.78 mole per mole of β-methylaspartate decomposed. During the first 20 min of incubation, a considerable amount of a radioactive compound having the mobility of α-

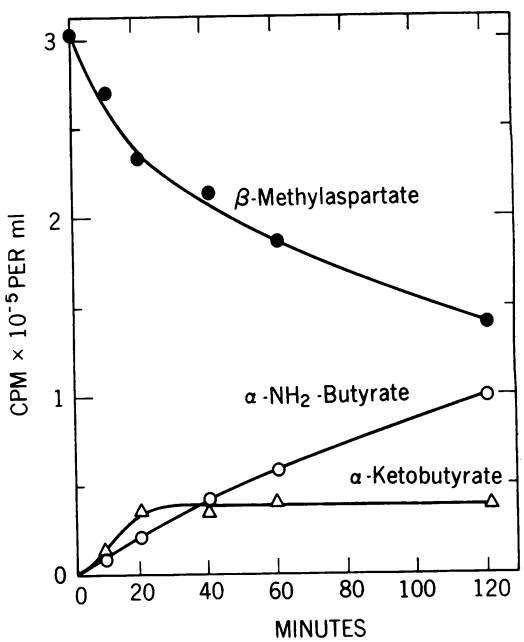

FIG. 1. *Time course of conversion of β-methylaspartate to α-aminobutyrate. The reaction mixture contained 5 μmoles of sodium ^{14}C-β-methylaspartate (specific activity, 6 × 10⁴ counts per min per μmole), 1 μmole of sodium α-ketoglutarate, 0.1 μmole of pyridoxal phosphate, 50 μmoles of potassium-phosphate buffer (pH 7.5), and 2.2 mg of extract protein in a total volume of 0.52 ml. Portions (50 μliters) were removed after the indicated periods of incubation at 37 C, and the reaction was stopped by heating the samples for 2 min at 100 C. After centrifugation, a 15-μliter portion was used for paper ionophoresis at pH 5.0 (70 min at 36 v per cm). The radioactive compounds on the ionophoretogram were located by radioautography, excised, and counted in a scintillation counter.*

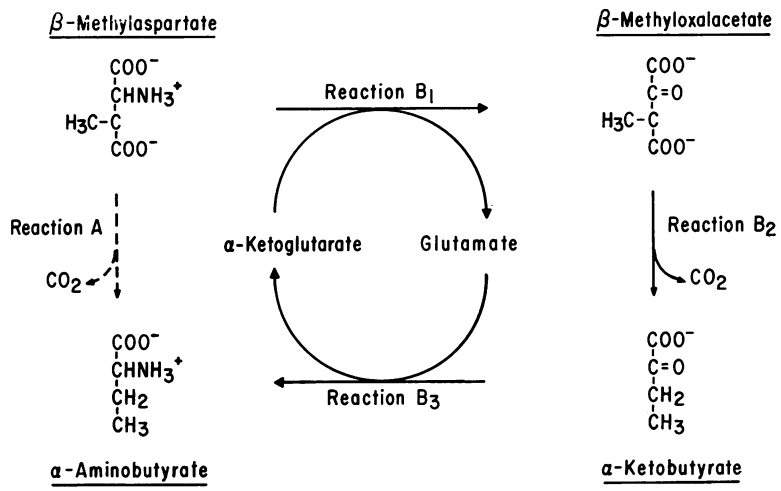

FIG. 2. *Postulated conversion of β-methylaspartate to α-aminobutyrate by P. putida. Both glutamate and β-methylaspartate can participate in the conversion of α-ketobutyrate to α-aminobutyrate (reaction B_3).*

ketobutyrate accumulated in the reaction mixture and remained essentially constant thereafter. The yield of this compound, calculated as α-ketobutyrate, was estimated to be 0.55 and 0.25 mole per mole of β-methylaspartate decomposed after 20 and 120 min of incubation, respectively.

Conversion of β-methyloxalacetate to α-aminobutyrate. When a reaction mixture containing 5 mM β-methyloxalacetate, 5 mM glutamate, 0.1 mM pyridoxal phosphate, and 4.3 mg of extract protein (from β-methylaspartate-grown cells) in 2 ml of 50 mM potassium phosphate buffer (*p*H 7.5) was incubated at 37 C, carbon dioxide and α-aminobutyrate were formed. Carbon dioxide evolution, followed manometrically, ceased after about 60 min of incubation, and the total yield was approximately 1 mole per mole of β-methyloxalacetate added. α-Aminobutyrate was identified by paper ionophoresis at *p*H 1.85; the yield was not determined. No α-aminobutyrate was detected when either glutamate or the extract was omitted from the reaction mixture, but rapid decarboxylation of β-methyloxalacetate occurred under both conditions.

DISCUSSION

The rates of oxidation of β-methylaspartate and related compounds by cell suspensions of the pseudomonad grown on β-methylaspartate, aspartate, or glutamate indicate that one or more inducible systems are involved in β-methylaspartate decomposition. The highest rate of β-methylaspartate oxidation was observed with β-methylaspartate-grown cells, an intermediate rate with aspartate-grown cells and a very low rate with glutamate-grown cells. It is not known if the inducible system is an enzyme or a transport system.

The decomposition of β-methylaspartate by extracts of β-methylaspartate-grown cells requires catalytic amounts of an α-ketoacid (e.g., α-ketoglutarate or pyruvate) and is stimulated by pyridoxal phosphate. The main products of β-methylaspartate decomposition are α-aminobutyrate and carbon dioxide. In addition, α-ketobutyrate accumulates in small amounts during β-methylaspartate decomposition. The cosubstrate, α-ketoglutarate or pyruvate, is at least partially converted to glutamate or alanine, respectively, presumably by transamination with β-methylaspartate.

The conversion of β-methylaspartate to α-aminobutyrate could occur by either or both of the paths (Fig. 2). One path (reaction A) is a direct β-decarboxylation of β-methylaspartate catalyzed by pyridoxal phosphate and an α-keto acid. A reaction of this type has been described by Novogrodsky and Meister (15). A second possible path (reactions $B_{1,2,3}$) involves the conversion of β-methylaspartate to β-methyloxalacetate by transamination with an α-keto acid, the enzymatic or nonenzymatic decarboxylation of β-methyloxalacetate to α-ketobutyrate, and conversion of the latter to α-aminobutyrate by transamination with either glutamate or β-methylaspartate. We favor the latter sequence of reactions because of (i) the relatively rapid formation of glutamate and alanine from α-ketoglutarate and pyruvate, respectively, in the presence of β-methylaspartate; (ii) the observed decarboxylation of β-methyloxalacetate and its conversion, in the presence of glutamate, to

α-aminobutyrate; and (iii) the evidence for the formation of α-ketobutyrate from β-methylaspartate. It may be noted that transaminations involving β-methylaspartate have been reported to be catalyzed by homogenates of normal and malignant animal tissues (8, 10, 14) as well as by *E. coli* (1).

Although under the conditions of our enzymatic experiments, α-aminobutyrate and alanine are the main products formed from β-methylaspartate and aspartate, respectively; the accumulation of these products has not been observed with intact cells. *In vivo*, the α-keto acids probably undergo oxidative decarboxylation and are then further oxidized via the tricarboxylic acid cycle. The amino group may be converted to ammonia by the action of glutamic dehydrogenase.

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