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Adenosine 5'-Triphosphate-Yielding Pathways of Branched-Chain Amino Acid Fermentation by a Marine Spirochete

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The metabolic pathways utilized by an obligately anaerobic marine spirochete (strain MA-2) to ferment branched-chain amino acids were studied. The spirochete catabolized L-leucine to isovaleric acid, L-isoleucine to 2-methylbutyric acid, and L-valine to isobutyric acid, with accompanying CO_2 production in each fermentation. Cell extracts of spirochete MA-2 converted L-leucine, L-isoleucine, and L-valine to 2-ketoisocaproic, 2-keto-3-methylvaleric, and 2-ketoisovaleric acids, respectively, through mediation of 2-ketoglutarate-dependent aminotransferase activities. The branched-chain keto acids were decarboxylated and oxidized to form isovaleryl coenzyme A, 2-methylbutyryl coenzyme A, and isobutyryl coenzyme A, respectively, in the presence of sulfhydryl coenzyme A and benzyl viologen. The acyl coenzyme A's were converted to acyl phosphates by phosphate branched-chain acyltransferase enzymatic activities. Branched-chain fatty acid kinase activities catalyzed formation of isovaleric, 2-methylbutyric, and isobutyric acids from isovaleryl phosphate, 2-methylbutyryl phosphate, and isobutyryl phosphate, respectively. Adenosine 5'-triphosphate was formed during conversion of branched-chain acyl phosphates to branched-chain fatty acids. The results indicate that conversion of L-leucine, L-isoleucine, and L-valine to branched-chain fatty acids by spirochete MA-2 results in adenosine 5'-triphosphate generation. The metabolic pathways utilized for this conversion involve amino acid aminotransferase, 2-keto acid oxidoreductase, phosphate acyltransferase, and fatty acid kinase activities.

An anaerobic marine spirochete (strain MA-2) ferments L-leucine, L-isoleucine, and L-valine, forming the branched-chain fatty acids isovaleric, 2-methylbutyric, and isobutyric acids as end products (5). The spirochete does not utilize branched-chain amino acids as growth substrates and grows only when glucose or another fermentable carbohydrate is available as a source of carbon and energy. Strain MA-2 does not require amino acids or branched-chain volatile fatty acids as growth factors (5). Numerous other anaerobic bacteria from various habitats also form significant amounts of isovaleric, 2-methylbutyric, and isobutyric acids (6). At least some of these bacteria resemble spirochete MA-2 in that they do not utilize leucine, isoleucine, and valine as carbon and energy sources for growth (1, 4). To our knowledge no studies have dealt with the enzymology of branched-chain amino acid fermentation by bacteria that form branched-chain volatile fatty acids.

Work described in an accompanying paper (5) showed that the viability of starved cells of spirochete MA-2 is prolonged when branched-chain amino acids are available, apparently because fermentation of the amino acids yields ATP that is utilized by the cells for maintenance energy. The present study was initiated (i) to investigate the enzymatic pathways of leucine, isoleucine, and valine fermentation in spirochete MA-2, and (ii) to determine whether, in fact, ATP was produced during fermentation of the branched-chain amino acids. Our intent was to assess the significance of branched-chain fatty acid production with regard to the overall physiology of spirochete MA-2 and, possibly, of other bacteria. In this report we describe the ATP-yielding enzymatic pathways of branched-chain amino acid fermentation present in spirochete MA-2.

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MATERIALS AND METHODS

Culture conditions. Spirochete MA-2 was isolated from intertidal marine mud as described previously (5). The bacteria were cultivated anaerobically in complex marine medium (MSM broth) (5). For enzyme assays, spirochete MA-2 was grown in 1.5-liter volumes

of MSM broth. The medium was inoculated with 9 ml (7×10^8 to 9×10^8 cells per ml) of a 24-h culture.

Preparation of cell extracts. Cells were harvested in late-logarithmic phase (after 24 h of growth) by centrifugation at 5°C for 20 min at 5,000 × *g*. Harvested cells were suspended in 60 ml of a 50 mM Tris-hydrochloride (pH 7.5 at 30°C)–4 mM dithiothreitol buffer solution in half-strength artifical seawater (5). The suspension was centrifuged, and the cells were washed by suspending them in 60 ml of fresh buffer and by sedimenting them by centrifugation. Finally, the washed cells were suspended in 7 ml of fresh buffer and stored at 5°C under N_2. The dithiothreitol was added to the buffer solution after the latter had been boiled and quickly chilled under N_2.

Spirochetes were disrupted by passing washed cell suspensions through a French pressure cell at 10,000 lb/in^2 (7×10^6 kg/m^2). Cellular debris was removed by centrifugation at 5°C for 20 min at 17,000 × *g*. The supernatant fluid was stored under N_2 before use in experiments. Cell extracts were used within 4 h in branched-chain amino acid aminotransferase and 2-keto acid oxidoreductase enzyme assays. Fresh cell extracts or extracts that had been stored frozen at −25°C were used in phosphate acyltransferase and fatty acid kinase assays. The protein content of cell extracts was determined by means of the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, Calif.).

Synthesis and identification of acyl phosphates. Acetyl phosphate (potassium lithium salt) was purchased from Sigma Chemical Co. (Saint Louis, Mo.). The remaining acyl phosphates were synthesized from their corresponding acyl chlorides and isolated as lithium salts by the method of Lipmann and Tuttle (11). Thin-layer chromatography was used to confirm the identities of synthesized acyl phosphates in the following manner. First, 2 mg of each acyl phosphate was converted to its acyl hydroxamate derivative by dissolving it in a solution of 0.9 ml of neutralized hydroxylamine (4 M hydroxylamine hydrochloride and 4 M KOH mixed in a 1:1 ratio) and 0.41 ml of water. After allowing 10 min for hydroxamates to form, 5- to 10-μl samples were spotted onto cellulose sheets (no. 13255; Eastman Kodak Co., Rochester, N.Y.). The solvent system was butanol-acetic acid-water (4:1:5). After drying, chromatograms were developed by spraying with 1.25% (wt/vol) $FeCl_3$ in 1.0 N HCl. The hydroxamates were identified by their R_f values and by comparison with standard hydroxamates prepared from commercially obtained acetyl phosphate, acetyl coenzyme A (CoA), isobutyryl-CoA, and isovaleryl-CoA (Sigma). To assess the purity of each synthesized compound, acyl hydroxamates were prepared by a modification of the hydroxamate method of Lipmann and Tuttle (10). Sample mixtures (1 ml) each containing acyl phosphate, 100 mM Tris-hydrochloride (pH 7.5 at 30°C), 350 mM neutralized hydroxylamine, and water were prepared and allowed to stand at room temperature for 10 min. Then 1 ml of 10% (wt/vol) trichloroacetic acid and 4 ml of 1.25% (wt/vol) $FeCl_3$ in 1.0 N HCl were added to each mixture. Absorbances at 540 nm of the hydroxamate samples were compared with absorbances of hydroxamate standards prepared from acetyl phosphate. The determination of purity of synthesized acyl phosphates was based on the assumption that the extinction coefficients of various acyl hydroxamate derivatives were identical to that of acetyl hydroxamate. On this basis the purities of synthesized compounds were found to range from 21% (for isovaleryl phosphate) to 71% (for propionyl phosphate). The synthesized acyl phosphates were relatively insoluble in water. For this reason, it was possible to include no more than 2.5 μmol of synthesized acyl phosphate substrate in 1-ml reaction mixtures prepared to assay fatty acid kinase and phosphate acyltransferase activities (see below).

Enzyme assays. All assays were performed under anaerobic conditions. Unless specified otherwise all assay mixtures were preincubated at room temperature in N_2 for at least 10 min before the initiation of reactions by addition of protein. Specific activities were determined in a range where activity was limited by protein concentration.

2-Ketoglutarate-dependent branched-chain amino acid aminotransferase activity was determined by following the formation of branched-chain keto acids. Assays were performed as described by Wong and Lessie (23), except that the 1-ml reaction mixtures were incubated for 30 min at 30°C in N_2. Reactions were terminated by the addition of 0.1 ml of 50% (wt/vol) trichloroacetic acid.

2-Keto acid oxidoreductase activity was measured spectrophotometrically (Gilford recording spectrophotometer) by following changes in absorbance of added electron acceptors in anaerobic septum cap cuvettes (A. H. Thomas, Philadelphia, Pa.). Reaction mixture components were dissolved in deoxygenated water (water that had been boiled for 1 min and rapidly cooled in N_2) and stored in N_2 before they were added to cuvettes. Anaerobic conditions were obtained by placing filled, uncapped cuvettes in a desiccator jar and evacuating for 10 min. The cuvettes were sealed with rubber septa, two syringe needles were inserted through each septum, and the cuvettes were then flushed with N_2 for 10 min. Under these conditions reduced benzyl viologen was not reoxidized by inverting the cuvettes, indicating the absence of oxygen in the system. The 3-ml reaction mixtures each contained the following: Tris-hydrochloride buffer (pH 7.5 at 30°C), 300 μmol; 2-keto acid, 30 μmol; sulfhydryl-CoA (CoA·SH), 0.15 μmol; thiamine pyrophosphate, 0.15 μmol; dithiothreitol, 9.0 μmol; and an electron acceptor. The electron acceptors tested (added to each reaction mixture) were as follows: benzyl viologen, 15 μmol; flavin adenine dinucleotide, 0.15 μmol; flavin mononucleotide, 0.15 μmol; and NAD, 0.15 μmol. Reactions were initiated by injecting 50 to 100 μl of cell extract into each capped cuvette with a Hamilton syringe (Hamilton Co., Reno, Ne).

Benzyl viologen reduction was determined by measuring the increase in absorbance at 540 nm at room temperature. The amount of benzyl viologen reduced was calculated by using a millimolar extinction coefficient of 6.2 (21). Flavin nucleotide reduction was measured by following decreases in absorbance at 450 nm, and NAD reduction was measured by following increases in absorbance at 340 nm. Benzyl viologen reduction took place only in the presence of cell extract and keto acid.

Phosphate acyltransferase activities were assayed by the method of Stadtman et al. (20) by measuring the hydrolysis of acyl phosphate in the presence of potassium arsenate, CoA·SH, and cell extract. Reaction mixtures contained the following: Tris-hydrochloride buffer (pH 8.0 at 30°C), 100 μmol; dithiothreitol, 3 μmol; CoA·SH, 0.2 μmol; and acyl phosphate, ca. 2.5 μmol (except for acetyl phosphate, 10 μmol). Water was added to a volume of 0.9 ml. Reaction mixtures were incubated at 30°C. Potassium arsenate (50 μmol, 0.1 ml) was added 5 min after reactions were initiated by the addition of protein. The reaction was terminated after 20 or 30 min by the addition of 350 μmol (0.2 ml) of neutralized hydroxylamine. After a 10-min incubation period to allow for formation of acyl hydroxamates, 1 ml of 10% (wt/vol) trichloroacetic acid was added to precipitate protein. Acyl hydroxamates were determined by measuring absorbance at 540 nm after the addition of 4 ml of 1.25% (wt/vol) $FeCl_3$ in 1.0 N HCl and removal of precipitated protein. Hydroxamate standards were prepared from acetyl phosphate. The specific activities assigned were based on the assumption that the extinction coefficients of acetyl hydroxamates were identical to those of other acyl hydroxamates, as described above. Separate experiments showed that the disappearance of each acyl phosphate was dependent on the addition of CoA·SH, potassium arsenate, and cell extract.

Fatty acid kinase activities were assayed in the reverse direction by measuring the formation of acyl phosphates from fatty acid and ATP by a modification of the method of Rose for acetate kinase (17). The 1-ml reaction mixtures each contained the following: Tris-hydrochloride buffer (pH 7.5 at 30°C), 50 μmol; $MnSO_4$, 4 μmol; ATP, 10 μmol; neutralized hydroxylamine, 350 μmol; and fatty acid (potassium salt), 60 μmol. Cell extracts were added last to initiate the reaction, and reaction mixtures were incubated for 30 min at 30°C in N_2. One milliliter of 10% (wt/vol) trichloroacetic acid was added to terminate the reaction, and precipitated protein was removed by centrifugation. Formation of acyl phosphates was measured by means of the hydroxamate method described above. Enzymatic activities were dependent on presence of ATP and cell extract.

Acyl hydroxamate products formed in reaction mixtures were separated and identified by thin-layer chromatography by a modification of the method of Stadtman and Barker (19). In other experiments the fatty acid kinase reactions were carried out as described above, but the reactions were terminated by the addition of 20 ml of 95% ethanol. Precipitated protein was removed by centrifugation, and the supernatant fluid was evaporated to a volume of 3 or 4 ml on a steam bath and finally to dryness at room temperature. The residue was suspended in 2 ml of absolute ethanol, and the supernatant fluid was evaporated to dryness. The residue was finally resuspended in 0.2 ml of absolute ethanol and used for chromatographic analysis. Samples (5 to 15 μl) were spotted onto cellulose sheets, and the acyl hydroxamates were separated and identified as described above.

Fatty acid kinase activities were also assayed in the forward direction by measuring the disappearance of acyl phosphate in the presence of ADP. The 1-ml reaction mixtures contained the following: Tris-hydrochloride buffer (pH 7.5 at 30°C), 50 μmol; $MnSO_4$, 4 μmol; ADP, 2 μmol; and acetyl phosphate, 10 μmol, or acyl phosphate, ca. 1.5 μmol. Cell extract was added to initiate the reactions. Reaction mixtures were incubated for 30 min at 30°C in N_2, and 350 μmol (0.2 ml) of neutralized hydroxylamine was added. After 10 min, 1 ml of 10% (wt/vol) trichloroacetic acid was added to precipitate protein. Disappearance of acyl phosphate was measured by the hydroxamate method described above.

In addition, fatty acid kinase activity was measured by monitoring synthesis of ATP from ADP in the presence of acyl phosphate and cell extract. Assay conditions were the same as those described above for assaying acetate kinase in the forward direction, except that $CHCl_3$ (0.25 ml) rather than neutralized hydroxylamine and trichloroacetic acid was added to stop the reaction. Extraction of ATP from the reaction mixture was performed as described by Lysko and Cox (13). ATP was determined by means of the firefly luciferin-luciferase assay (13), using 10 μl of a 1:100 dilution of the resuspended reaction mixture. A standard curve was constructed from known concentrations of ATP.

Other procedures. Coenzyme A was removed from cell extracts by the method of Stadtman et al. (20) as follows. Cell extracts were added to Amberlite IRA-400 resin (20–50 mesh) (Mallinckrodt Chemical Works, St. Louis, Mo.) in a ratio of 1:0.5 (vol/vol). The suspensions were stirred for 15 min in N_2 and centrifuged to separate resin from cell extracts. Then extracts were filtered through Whatman no. 1 filter paper to remove any remaining resin.

RESULTS

Branched-chain fatty acid formation. Enzymatic activities functioning in the conversion of L-leucine, L-isoleucine, and L-valine to isovaleric, 2-methylbutyric, and isobutyric acids, respectively, were detected in extracts of spirochete MA-2 cells. The enzymatic activities detected were branched-chain amino acid aminotransferase(s), branched-chain keto acid oxidoreductase(s), phosphate branched-chain acyltransferase(s), and branched-chain fatty acid kinase(s).

Amino acids were converted to their corresponding branched-chain keto acids by L-leucine, L-isoleucine, and L-valine aminotransferase activities. The specific activities were 275, 269, and 208 nmol of branched-chain keto acid formed per min per mg of protein, respectively. These conversions were dependent on addition of 2-ketoglutarate. Cell extracts catalyzed a CoA·SH-dependent oxidation of branched-chain keto acids in the presence of benzyl viologen (Table 1). No enzymatic activity (reduction) was observed with flavin mononucleotide, flavin adenine dinucleotide, or NAD as electron acceptors for keto acid oxidation. Phosphate branched-

TABLE 1. *Branched-chain 2-keto acid oxidoreductase activities in cell extracts of spirochete MA-2*

Reaction	Sp act[a]	
	With CoA·SH	Without CoA·SH
2-Ketoisocaproic acid oxidoreductase	141	22
2-Keto-3-methylvaleric acid oxidoreductase	65	10
2-Ketoisovaleric acid oxidoreductase	130	16

[a] Expressed as nanomoles of benzyl viologen reduced per minute per milligram of protein.

chain acyltransferase activities in cell extracts were assayed by measuring the disappearance of different acyl phosphates in the presence of potassium arsenate. Enzymatic activities that catalyze the interconversion of branched-chain acyl-CoA's with corresponding branched-chain acyl phosphates were detected (Table 2).

Cell extracts catalyzed the formation of branched-chain acyl hydroxamates from branched-chain fatty acids and ATP (Table 2). It seemed likely that branched-chain fatty acid kinases were catalyzing these reactions, inasmuch as cell extracts were found to have phosphate branched-chain acyltransferase activities. However, it was possible that the conversion of branched-chain fatty acids and ATP to acyl hydroxamates was catalyzed by acyl-CoA:acetate-CoA transferase (EC 2.8.3.8) (18, 22). Alternatively, enzymatic activities similar to AMP-forming acetyl-CoA synthetase (EC 6.2.1.1) or ADP-forming acetyl-CoA synthetase (EC 6.2.1.13), but specific for branched-chain substrates, could be involved in the formation of branched-chain acyl hydroxamates (7, 15). To exclude these possibilities we carried out experiments with cell extracts from which exogenous CoA had been removed by treatment with Amberlite ion-exchange resin. Branched-chain acyl hydroxamates were formed from ATP and fatty acid in the presence of CoA-depleted cell extracts. Addition of exogenous CoA·SH to Amberlite-treated or nontreated cell extracts did not stimulate these enzymatic activities. Furthermore, branched-chain fatty acid kinase activities were assayed and detected in the forward direction by measuring the disappearance of acyl phosphate as well as the formation of ATP in the presence of ADP and cell extract. These experiments (results not shown) indicated that cell extracts catalyzed the formation of ATP and branched-chain fatty acid from acyl phosphate and ADP. On the basis of these results it was concluded that branched-chain fatty acid kinases mediated the conversion of acyl phosphates and ADP to fatty acids and ATP.

The hydroxamic acid derivatives of acyl phosphates formed in fatty acid kinase reaction mixtures had the following R_f values when separated by thin-layer chromatography on cellulose sheets: isobutyryl hydroxamate, 0.88; isovaleryl hydroxamate, 0.92; and 2-methylbutyryl hydroxamate, 0.92. Acetyl hydroxamate (derived from acetyl phosphate formed by acetate kinase in the presence of acetic acid, ADP, and cell extract) had an R_f value of 0.64.

Acetic acid formation. Enzymatic activities that catalyzed the conversion of pyruvic acid to acetic acid were detected in extracts of spirochete MA-2 cells. A pyruvic acid oxidoreductase activity catalyzed the CoA·SH-dependent reduction of benzyl viologen (specific activity, 152 nmol of benzyl viologen reduced per min per mg of protein). No enzyme activity was observed with flavin mononucleotide, flavin adenine dinucleotide, or NAD as an electron acceptor. Furthermore, phosphate acetyltransferase and acetate kinase activities were present in cell extracts. The specific activities were 1,660 and 788 nmol/min per mg of protein, respectively.

Substrate specificity. It seemed of interest to determine whether cell extracts of spirochete MA-2 catalyzed the formation of fatty acids from keto acids other than pyruvic and branched-chain keto acids. 2-Ketobutyric, 2-ketocaproic, and 2-ketovaleric acids were oxidized in the presence of CoA·SH and cell extract with benzyl viologen as an electron acceptor. Activities were comparable to those reported herein for pyruvic and branched-chain keto acid oxidoreductase(s). Phosphate acyltransferase and fatty acid kinase enzymes that were active with straight-chain substrates containing three, four, or five carbon atoms were also present in cell extracts (Table

TABLE 2. *Phosphate branched-chain acyltransferase[a] and branched-chain fatty acid kinase[b] activities in cell extracts of spirochete MA-2*

Reaction	Sp act[c]
Phosphate isovaleryltransferase	35 ± 14.2
Phosphate 2-methylbutyryltransferase	9 ± 2.5
Phosphate isobutyryltransferase	138 ± 56.2
Isovalerate kinase	36 ± 2.4
2-Methylbutyrate kinase	19 ± 2.9
Isobutyrate kinase	38 ± 5.7

[a] Specific activity expressed as nanomoles of acyl phosphate used per minute per milligram of protein.

[b] Specific activity expressed as nanomoles of acyl phosphate formed per minute per milligram of protein.

[c] Results represent the average of triplicate experiments ± standard deviation.

3). The hydroxamic acid derivatives of straight-chain acyl phosphates (formed in fatty acid kinase reaction mixtures) had the following R_f values when separated by thin-layer chromatography: propionyl hydroxamate, 0.78; butyryl hydroxamate, 0.87; and valeryl hydroxamate, 0.92.

DISCUSSION

Data presented in this paper show that marine spirochete MA-2 has the ability to generate ATP by substrate level phosphorylation as a result of the fermentations of L-leucine, L-isoleucine, and L-valine. The conversion of each branched-chain amino acid to its corresponding fatty acid end product by spirochete MA-2 involves four enzymatic activities (Fig. 1). To our knowledge there are no reports of previous work directly demonstrating the specific enzymatic steps involved in leucine, isoleucine, and valine fermentation. In an accompanying paper (5) we describe and discuss experiments which indicate that (i) spirochete MA-2 does not utilize branched-chain amino acids as sole carbon and energy sources for growth, and (ii) ATP derived from the metabolism of branched-chain amino acids serves as a source of maintenance energy for spirochete MA-2 when growth substrates are not available.

Some anaerobic bacteria that catabolize branched-chain amino acids with formation of fatty acids also have the ability to convert branched-chain volatile fatty acids to amino acids which they use in the synthesis of cell material (1–3, 16). It has been shown, for example, that growing cells of *Megasphaera* (*Peptostreptococcus*) *elsdenii* and *Bacteroides ruminicola* ferment valine, producing isobutyric acid, and also convert isobutyric acid to valine found in cellular protein (2, 3). The pathway we report here for the catabolism of L-valine to isobutyric acid (Fig. 1) is the reverse of a pathway proposed by Allison and Peel (3) for the biosynthesis of valine from isobutyric acid. Thus, it seems likely that these rumen bacteria ferment L-valine and possibly other amino acids by pathways similar to those used by spirochete MA-2.

We did not determine whether growing cells of spirochete MA-2 had the ability to convert exogenous branched-chain volatile fatty acids into cellular amino acids. Spirochete MA-2 does not require this biosynthetic ability inasmuch as it does not require exogenous volatile fatty acids for growth. It is possible, however, that energy expenditures for biosynthesis of branched-chain amino acids from volatile fatty acids are smaller than those for biosynthesis of leucine, isoleucine, and valine from glucose or other fermentable carbohydrates.

TABLE 3. *Specificity of phosphate acyltransferase and fatty acid kinase activities in cell extracts of spirochete MA-2*

Substrate	Phosphate acyltransferase sp act[a]	Fatty acid kinase sp act[b]
Propionyl phosphate	1,748	
Butyryl phosphate	56	
Valeryl phosphate	52	
Propionate		821
Butyrate		80
Valerate		75

[a] Expressed as nanomoles of acyl phosphate used per minute per milligram of protein.

[b] Expressed as nanomoles of acyl phosphate formed per minute per milligram of protein.

We have shown that spirochete MA-2 converts to branched-chain fatty acids only a relatively small proportion of the total amount of branched-chain amino acid available in growth media (5). It is not clear why the amount of branched-chain volatile fatty acid formed by strain MA-2 is so small as compared with the amount of acetic acid and other fermentation products formed from glucose. However, this observation is consistent with our finding that the levels of phosphate branched-chain acyltransferase and branched-chain fatty acid kinase activities measured in cell extracts were low in comparison with similar activities for formation of acetic acid from acetyl-CoA. In general the specific activities of branched-chain keto acid oxidoreductase(s) were approximately equal to the specific activity of pyruvic acid oxidoreductase in spirochete MA-2. Specific activities of branched-chain amino acid aminotransferase(s) were comparable to those measured in other bacteria (9, 23). In addition to their role in the catabolism of branched-chain amino acids, aminotransferase enzymes may be used by spirochete MA-2 for biosynthesis of amino acids from glucose (14). The cellular lipid content of strain MA-2 has not been examined. However, lipids of other free-living spirochetes have been reported to contain branched long-chain fatty acids (12). Thus, it is possible that branched-chain keto acid oxidoreductase activities in spirochete MA-2 are involved in the biosynthesis of branched long-chain fatty acids from glucose (8).

The oxidation of 2-keto acids by spirochete MA-2 required the presence of benzyl viologen as an electron acceptor. Neither FMN, FAD, nor NAD substituted as electron acceptors under the assay conditions used in these experiments. It is likely that growing cells use electrons generated by the cleavage of 2-keto acids in the

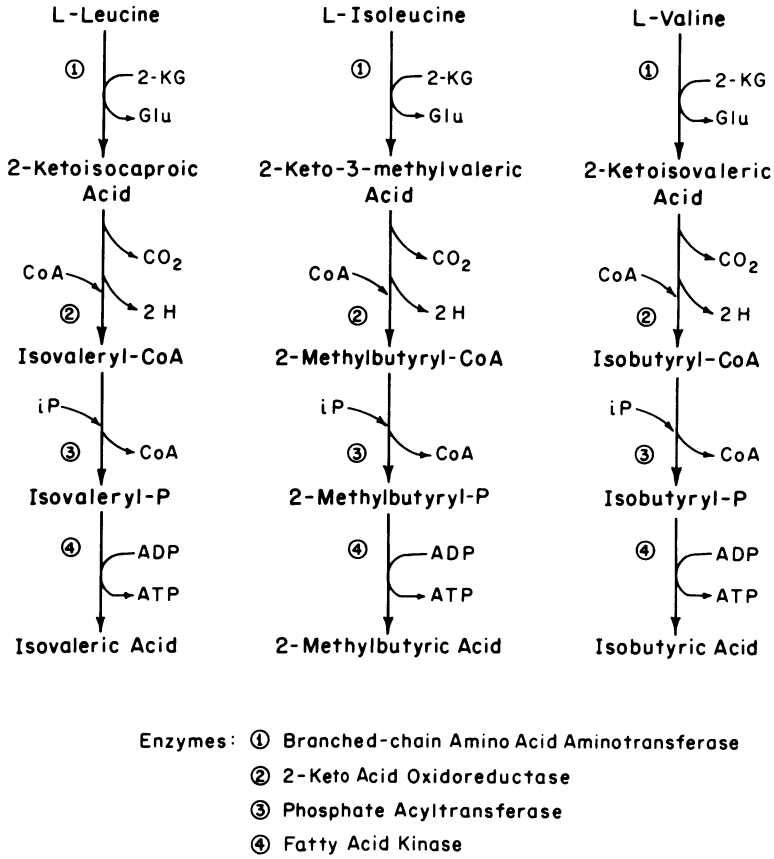

FIG. 1. *Enzymatic pathways of branched-chain amino acid fermentation by spirochete MA-2. 2-KG, 2-Ketoglutarate; Glu, glutamate.*

formation of hydrogen gas since this is a major fermentation product of strain MA-2 (5). It is not known what natural electron acceptor mediates the transfer of electrons between keto acids and hydrogen. The possibility that the oxidation of 2-keto acids was linked to ferredoxin was not examined.

Since branched-chain amino acid fermentation in spirochete MA-2 was found to be an ATP-yielding pathway, it seemed possible that branched-chain amino acid aminotransferase activities might be subject to energy-linked regulation in a fashion analogous to that observed for threonine dehydrases from *Escherichia coli* and *Clostridium tetanomorphum* (24). However, neither AMP or ADP (10 mM) was found to influence branched-chain amino acid aminotransferase activities in spirochete cell extracts.

The enzymatic steps utilized by spirochete MA-2 for the formation of acetic acid from pyruvic acid are analogous to those responsible for the formation of branched-chain fatty acids from branched-chain keto acids. It may be speculated that the pyruvic acid oxidoreductase, phosphate acetyltransferase, and acetate kinase enzymes which are present at high levels in spirochete MA-2 cells have relatively broad substrate specificities and thus are able to catalyze reactions involving branched-chain substrates. Additional work is required to demonstrate whether the various steps in the fermentations of pyruvic acid, leucine, isoleucine, and valine are catalyzed by single or multiple enzymes.

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