

Role of branched-chain 2-oxo acid dehydrogenase and pyruvate dehydrogenase in 2-oxobutyrate metabolism

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Purified branched-chain 2-oxo acid dehydrogenase (BCODH) and pyruvate dehydrogenase (PDH) had apparent K_m values (μM) for 2-oxobutyrate of 26 and 114, with a relative V_{max} (% of V_{max} for 3-methyl-2-oxobutyrate and pyruvate) of 38 and 45% respectively. The phosphorylation state of both complexes in extracts of mitochondria from rat liver, kidney, heart and skeletal muscle was shown to influence oxidative decarboxylation of 2-oxobutyrate. Inhibitory antibodies to BCODH and an inhibitor of PDH (3-fluoropyruvate) were used with mitochondrial extracts to determine the relative contribution of both complexes to oxidative decarboxylation of 2-oxobutyrate. Calculated rates of 2-oxobutyrate decarboxylation in mitochondrial extracts, based on the kinetic constants given above and the activities of both complexes, were the same as the measured rates. Hydroxyapatite chromatography of extracts of mitochondria from rat liver revealed only two peaks of oxidative decarboxylation of 2-oxobutyrate, with one peak associated with PDH and the other with BCODH. Competition studies with various 2-oxo acids revealed a different inhibition pattern with mitochondrial extracts from liver compared with those from heart or skeletal muscle. We conclude that both intramitochondrial complexes are responsible for oxidative decarboxylation of 2-oxobutyrate. However, the BCODH is probably the more important complex, particularly in liver, on the basis of kinetic analyses, activity or phosphorylation state of both complexes, competition studies, and the apparent physiological concentration of pyruvate, 2-oxobutyrate and the branched-chain 2-oxo acids.

INTRODUCTION

Threonine and methionine are primarily catabolized by liver, although other tissues can metabolize these essential amino acids (Miller, 1962; Anguilar *et al.*, 1974; Kang-Lee & Harper, 1978; Mitchell & Benevenga, 1978). Although both amino acids have two catabolic pathways, each of them can be converted into 2-oxobutyrate (Meister, 1965; Finkelstein & Martin, 1984; Livesey, 1984). Further catabolism of 2-oxobutyrate gives three possible products: 2-aminobutyrate, 2-hydroxybutyrate, or, from oxidative decarboxylation, propionyl-CoA (Meister, 1965; Ciman & Siliprandi, 1968; Bremer, 1969; Landaas & Pettersen, 1975; Steele, 1982). The intramitochondrial oxidative decarboxylation of 2-oxobutyrate requires CoA, NAD^+ and thiamin pyrophosphate (Siliprandi & Ciman, 1969; Steele *et al.*, 1984), as does the oxidative decarboxylation of pyruvate and the branched-chain 2-oxo acids formed from the branched-chain amino acids. Pyruvate and the branched-chain 2-oxo acids are oxidatively decarboxylated by their respective intramitochondrial multienzyme complexes, pyruvate dehydrogenase (PDH; EC 1.2.4.1 + 2.3.1.12 + 1.6.4.3) and branched-chain 2-oxo acid dehydrogenase (BCODH); however, no 2-oxobutyrate dehydrogenase has been isolated. It has been suggested numerous times (e.g. Bremer, 1969; Kanzaki *et al.*, 1969; Newsholme & Leech, 1983; McGilvery, 1983; Steel *et al.*, 1984) that PDH is responsible, at least

partially or solely, for the oxidative decarboxylation of 2-oxobutyrate; although the possibility of another intramitochondrial enzyme (Bremer, 1969; Borud & Pettersen, 1982; Steele *et al.*, 1984) or a specific 2-oxobutyrate dehydrogenase (Yang & Roth, 1985) has been suggested.

The present study demonstrates that oxidative decarboxylation of 2-oxobutyrate is attributable to both BCODH and PDH, based on several criteria: kinetic analyses of the purified complexes; the use of specific inhibitors of both complexes; alterable rates of 2-oxobutyrate decarboxylation in mitochondrial extracts by treatment with phosphoprotein phosphatase and ATP; hydroxyapatite-chromatographic separation of mitochondrial extracts; and predictability of the rate of oxidative decarboxylation of 2-oxobutyrate, after various treatments, based on the measured activities of PDH and BCODH. We conclude, however, that hepatic BCODH is the complex predominantly responsible for oxidative decarboxylation of 2-oxobutyrate on the basis of the above data, competition studies between various 2-oxo acids with mitochondrial extracts, phosphorylation state of both complexes in liver, and the apparent physiological concentrations of pyruvate, 2-oxobutyrate and the branched-chain 2-oxo acids. The data, taken together, also appear definitively to show that there is no separate and specific enzyme for the oxidative decarboxylation of 2-oxobutyrate.

Abbreviations used: BCODH, branched-chain 2-oxo acid dehydrogenase; PDH, pyruvate dehydrogenase.

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EXPERIMENTAL

Materials

DL-2-Amino[1-¹⁴C]butyrate was from ICN Radiochemicals; [1-¹⁴C]pyruvate was from Amersham; BCODH was prepared from rabbit liver (Paxton & Harris, 1982); PDH was from bovine heart (Sigma Chemical Co.); and unless indicated all other reagents were from Sigma. 2-Oxo[1-¹⁴C]butyrate was prepared from DL-2-amino[1-¹⁴C]butyrate (40 Ci/mol) by incubation in 0.1 M-Tris/HCl (pH 7.2), with 25000 units of catalase and 3 units each of D- and L-amino acid oxidase (final volume 2 ml). The medium was gassed for 10 min with O₂/CO₂ (19:1) before start of incubation. The reaction (3 h at 37 °C) was terminated by addition of 6 M-HCl until the medium was at pH 2. 2-Oxo[1-¹⁴C]butyrate was isolated by chromatography on Dowex 50 (H⁺ form), with elution by 1 mM-HCl. The yield of 2-oxo[1-¹⁴C]butyrate was approx. 70%, and purity was > 95% based on t.l.c. on silica gel with as solvent butan-1-ol-saturated water with 5% (v/v) formic acid.

Preparation and characterization of antibodies to BCODH

Antibodies to homogeneous rabbit liver BCODH were raised in a goat. Serum for isolation of IgG from the immunized goat was obtained when the titre, based on BCODH inhibition, reached a maximum. The IgG fraction was isolated from serum by (NH₄)₂SO₄ fractionation, and chromatography on DEAE-Sephacel and on CM-Affi-Gel (Bio-Rad). The final IgG fraction (98 mg/ml) was stored at -70 °C in 0.03 M-potassium phosphate, pH 7.5. SDS/polyacrylamide-gel electrophoresis (Paxton & Harris, 1982) revealed only two subunits, with *M_r* values consistent with the subunits of IgG.

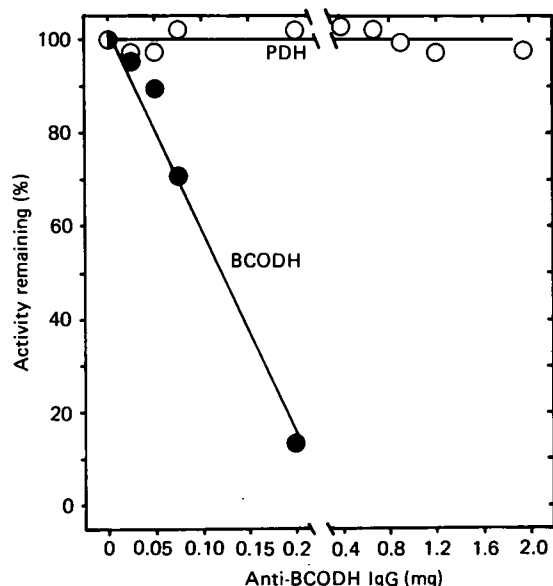


Fig. 1. Inhibition of BCODH by antibodies raised against BCODH

Sources of purified enzymes and antibodies are given in the Experimental section. Incubation of antibodies (in amount given) was for 15 min at 30 °C with either 5.5 µg of PDH (○) or 5.4 µg of BCODH (●). Activity was measured spectrophotometrically as described in the Experimental section.

The IgG inhibited purified BCODH (pre-immune serum did not inhibit; results not shown) but not purified PDH (Fig. 1) or 2-oxoglutarate dehydrogenase (results not shown). The titre (µg of IgG needed to inhibit 1 µg of BCODH) was not affected by the amount of complex (results not shown) or by the phosphorylation state of the complex (Fig. 2). The titre was determined (Rogers & Rudney, 1982) by measuring the remaining activity (y axis) with at least five different amounts of IgG (x axis). Linear regression gave the x-axis intercept, which was the titre.

Preparation and treatment of mitochondria

Mitochondria were isolated, as described, from rat liver, kidney (Johnson & Lardy, 1967), heart (Davis, 1965) and skeletal muscle (Scislowski, 1977). Isolated mitochondria were pelleted and stored frozen at -70 °C. Mitochondrial extracts were prepared by vigorously mixing mitochondrial pellets (2-6 mg of protein/ml) in ice-cold 50 mM-Hepes/KOH containing 3 mM-EDTA, 5 mM-dithiothreitol, 1% (v/v) Triton X-100, 0.5 mM-7-amino-1-chloro-3-L-tosylamidoheptan-2-one, 0.5 µM-pepstatin A, 0.5 µM-leupeptin, 1.0 µg of aprotinin/ml, 0.1 mg of trypsin inhibitor from turkey egg white/ml,

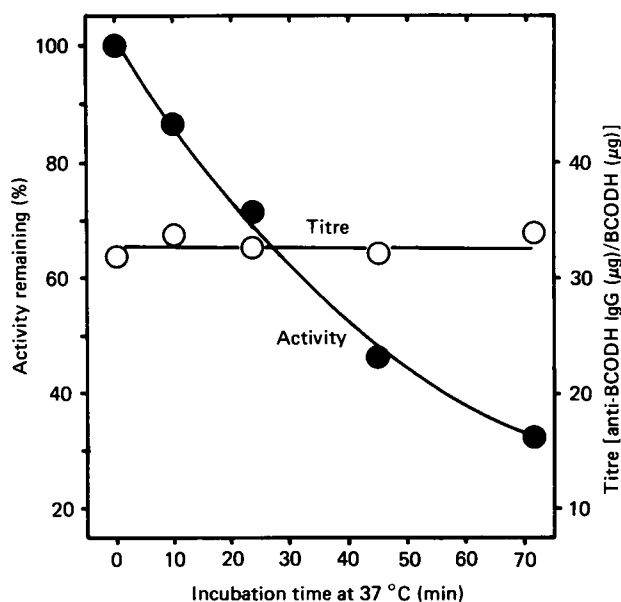


Fig. 2. Influence of phosphorylation state of BCODH on inhibition by antibodies

Purified BCODH was phosphorylated as previously described (Paxton & Harris, 1984a) with 0.25 mM-ATP. At the indicated times a sample was removed from the phosphorylation assay and made to 20 mM-EDTA (potassium salt; pH 7.5) and 1 mM-ADP to inhibit BCODH kinase. Samples (approx. 6 µg of BCODH) were mixed with either no antibody or five different amounts of antibody (30-140 µg), incubated at 30 °C for 20 min, and BCODH activity was determined as described in the Experimental section. The titre (µg of IgG to inhibit 1 µg of BCODH) was determined as described in the Experimental section. Each point (●, activity; ○, titre) represents the mean for at least three determinations, with the s.d. less than the diameter of the point. There was no difference in the titres with different degrees of phosphorylation, based on analysis of variance.

0.5% (v/v) dialysed bovine serum (pH 7.5 at 20 °C) and, immediately after mixing, made to 0.5 mM-phenylmethanesulphonyl fluoride. The extract, maintained on ice, was either made to 50 mM-KF, 25 mM-potassium phosphate (pH 7.5) and 0.5% dialysed bovine serum or was treated as described below. For activation by isolated broad-specificity phosphoprotein phosphatase (Harris *et al.*, 1982; Paxton & Harris, 1984a), hereafter termed 'phosphatase', a sample of the extract was made to 5 mM-MgCl₂, 0.5% dialysed bovine serum and approx. 1–2% (v/v) of isolated phosphatase (16.8 mg/ml). This mixture was incubated for approx. 20 min at 30 °C. This was sufficient time, with these conditions, to obtain maximum activity with any of the 2-oxo acid substrates. Phosphatase treatment was stopped at the indicated times by addition of KF and potassium phosphate (pH 7.5) to concentrations of 50 and 25 mM respectively. In those experiments where ATP was added, a portion of the above mixture (after addition of KF and potassium phosphate) was made to 1 mM-ATP and incubated for another 10 min at 30 °C. Inactivation by added ATP was terminated by the addition of EDTA potassium salt; (pH 7.5) to 10 mM. Treatment of mitochondrial extracts with antibodies against BCODH was either before or after phosphatase treatment as indicated. Approx. 1 µg of IgG was added per µl of mitochondrial extract, and the mixture was incubated at 20 °C for 15 min. After the above treatments, extracts were always placed on ice and assayed immediately.

Assay of oxidative decarboxylation of 2-oxo acids

A spectrophotometric assay at 340 nm (production of NADH) was used for determination of the activities of purified BCODH and PDH, and when assaying mitochondrial extracts with 3-methyl-2-oxobutyrates. A radiometric assay for decarboxylation of [1-¹⁴C]pyruvate and 2-oxo[1-¹⁴C]butyrate was used for mitochondrial extracts. This assay was conducted in a 25 ml Erlenmeyer flask fitted with a serum cap with a hanging well containing fluted filter paper. Phenethylamine (Eastman Kodak; approx. 0.3 ml) was placed in the hanging well. The reaction was terminated by addition of 1 ml of 2 M-acetic acid, and incubated at 42 °C for 1 h to collect CO₂. The hanging well was removed, placed in 5 ml of ScintiVerse E (Fisher Scientific Co.), and radioactivity determined. A blank was always determined in the same manner, except that acid was added before substrate. The assay, either spectrophotometric or radiometric, was at 30 °C in a total volume of 1 ml at pH 7.5 with 30 mM-potassium phosphate, 2 mM-MgCl₂, 0.4 mM-thiamin pyrophosphate, 0.4 mM-CoA, 3 mM-NAD⁺, 0.1% Triton X-100, 2 mM-dithiothreitol, 2 units of pig heart lipoamide dehydrogenase and 1 mM 2-oxo acid substrate (except for kinetic studies). All reactions were started by addition of substrate. Specific radioactivities of [1-¹⁴C]pyruvate and 2-oxo[1-¹⁴C]butyrate were between 50 and 200 c.p.m./nmol. Oxidative decarboxylation of pyruvate and 2-oxobutyrates was also determined spectrophotometrically before radiometric determination under the above assay conditions, but with 10 mM-NAD⁺ and 3 mM-oxamate, which minimized the interference by lactate dehydrogenase. These determinations were used to estimate the amount of extract and assay time for the radiometric assay. The radiometric and spectrophotometric assays were linear with time and increasing amounts of extract until approx. 50–60 nmol of product

was formed (results not shown). All assays were conducted with an amount of the mitochondrial extract for a given time (5–10 min) to generate < 50 nmol of product. One unit of activity is 1 µmol of product produced/min at 30 °C. Protein determinations and kinetic analysis were as previously described (Paxton & Harris, 1982).

RESULTS

Kinetic characterization of purified BCODH and PDH

Kinetic analyses of purified rabbit liver BCODH and bovine heart PDH were performed with the spectrophotometric assay with varied concentrations of all three 2-oxo acids. This PDH preparation showed no activity towards 2-oxoglutarate or 3-methyl-2-oxobutyrates (1 mM). The kinetic parameters (apparent K_m and V_{max}) are given for all three 2-oxo acids with both complexes (Table 1). BCODH has previously been shown to utilize pyruvate (Pettit *et al.*, 1978; Paxton & Harris, 1982), although the apparent K_m was very high and the V_{max} was approx. 20% of that with 3-methyl-2-oxobutyrates. The apparent K_m of 2-oxobutyrates (26 µM) with BCODH was similar to that of 3-methyl-2-oxobutyrates (35 µM), although the V_{max} was only 38% of that with 3-methyl-2-oxobutyrates. These data are similar to those obtained previously (Pettit *et al.*, 1978). PDH did not oxidatively decarboxylate 3-methyl-2-oxobutyrates. This complex had an apparent K_m and V_{max} with 2-oxobutyrates that was 2-fold higher and 55% less, respectively, than those with pyruvate. The apparent K_m for 2-oxobutyrates was approx. 4-fold greater with PDH than with BCODH. Thus 2-oxobutyrates are a better substrate for BCODH than for PDH. Also, isolated 2-oxoglutarate dehydrogenase did not utilize 2-oxobutyrates (1 mM), as previously described (Kanzaki *et al.*, 1969).

Regulation of oxidative decarboxylation of 2-oxobutyrates in mitochondrial extracts by phosphorylation and dephosphorylation

Only two intramitochondrial enzymes, PDH and BCODH, are known to be regulated by a phosphorylation mechanism (Hughes & Halestrap, 1981). Treatment of liver mitochondrial extracts with a phosphatase increased oxidative decarboxylation of both pyruvate (PDH activity) and 2-oxobutyrates, but not 3-methyl-2-oxobutyrates (BCODH activity; Fig. 3). After the activities reached a maximum, and the phosphatase was inhibited by added KF and potassium phosphate, incubation of the extract with ATP led to inhibition of the oxidative decarboxylation of all three 2-oxo acids. Results of a similar nature, although with different relative activities, different degrees of activation by phosphatase treatment, and different degrees of inhibition by ATP, were seen with mitochondrial extracts from rat kidney, heart and skeletal muscle (results not shown). Thus oxidative decarboxylation of 2-oxobutyrates appears to be regulated by a phosphorylation mechanism and to be associated with the phosphorylation state of both PDH and BCODH.

Influence of BCODH and PDH inhibitors on rates of 2-oxobutyrates decarboxylation and predictability of these rates in mitochondrial extracts

Analysis of the relative contributions of BCODH and PDH, or possibly another enzyme, to oxidative decar-

Table 1. Kinetic constants for isolated BCODH and PDH

Apparent K_m values are given as μM and V_{max} as $\mu\text{mol/min per mg}$ of protein. Kinetic parameters were determined as described in the Experimental section and are given as means \pm S.E.M.

Enzyme	Substrate					
	Pyruvate		2-Methyl-2-oxobutyrate		2-Oxobutyrate	
	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}
BCODH	820 ± 80	2.20 ± 0.07	35.2 ± 2.3	10.6 ± 0.3	25.8 ± 1.2	4.01 ± 0.06
(% of V_{max})		20.8%		100%		37.8%
PDH	50.8 ± 1.5	0.91 ± 0.01	0.0	0.0	114 ± 7.1	0.41 ± 0.01
(% of V_{max})		100%				45.4%

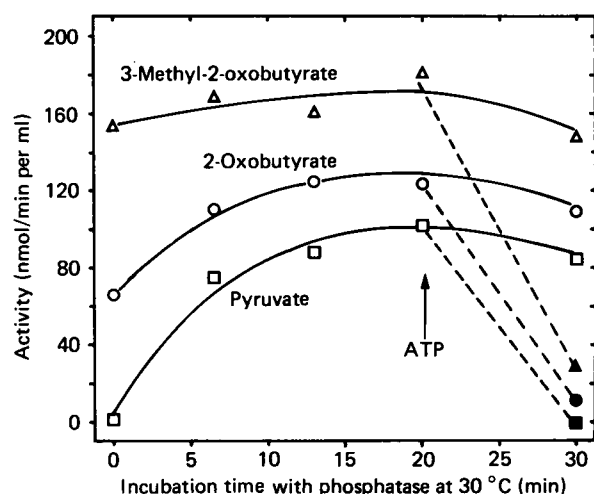


Fig. 3. Time course of phosphatase and ATP treatments on oxidative decarboxylation of 3-methyl-2-oxobutyrate (Δ), 2-oxobutyrate (\circ) and pyruvate (\square)

Liver mitochondrial extracts (essentially lactate dehydrogenase-free), spectrophotometric assays (minus oxamate and with 3 mM-NAD⁺), phosphatase and ATP treatments were as described in the Experimental section. ATP was added at 20 min. Activities (nmol/min per ml at 30 °C) with the indicated 2-oxo acid (1 mM) represented by the black symbols are after ATP addition.

boxylation of 2-oxobutyrate in extracts of mitochondria from various tissues was done by using inhibitory antibodies to the former, and a potent competitive inhibitor, 3-fluoropyruvate (Bisswanger, 1981), of the latter. 3-Fluoropyruvate at 50 μM did not inhibit the oxidative decarboxylation of 1 mM-3-methyl-2-oxobutyrate by purified BCODH, although with purified PDH it inhibited oxidative decarboxylation of 2-oxobutyrate more than of pyruvate (both at 1 mM; results not shown). The greater inhibition of oxidative decarboxylation of 2-oxobutyrate compared with that of pyruvate by 3-fluoropyruvate would be expected, since the apparent K_m of 2-oxobutyrate was greater than for pyruvate. Oxidative decarboxylation of 2-oxobutyrate apparently was due primarily to BCODH in liver mitochondria

before phosphatase treatment (Table 2; also see Fig. 3), since inhibitory antibodies to this complex greatly diminished BCODH activity (85%) and 2-oxobutyrate decarboxylation (65%), with only a slight inhibition (25%) of pyruvate decarboxylation. The latter effect of antibody treatment was expected, owing to the activity of BCODH towards pyruvate (calculated to be approx. 4.7 munits/mg of mitochondria protein, based on 12% of the activity of BCODH; see below). After phosphatase treatment, when decarboxylation of pyruvate increased by 80% (apparently owing to dephosphorylation of PDH), decarboxylation of 2-oxobutyrate was inhibited more by 3-fluoropyruvate (31%) and less by antibody treatment (50%) compared with values before phosphatase treatment. Also, after the phosphatase treatment had been inhibited by potassium phosphate and KF, added ATP led to decreased oxidative decarboxylation of all three 2-oxo acids. Treatment of mitochondrial extracts with antibody and 3-fluoropyruvate decreased the rate of decarboxylation of all three 2-oxo acids to essentially zero (results not shown). Assuming that PDH and BCODH are the only two enzymes responsible for oxidative decarboxylation of 2-oxobutyrate, as indicated by the above data, then the expected rate of oxidative decarboxylation of 2-oxobutyrate can be calculated by using the kinetic constants obtained with the isolated complexes (see above) and the activities of BCODH and PDH. Thus the expected rate of 2-oxobutyrate decarboxylation should be 0.38 times BCODH activity plus 0.43 times the rate of pyruvate decarboxylation (PDH plus BCODH activity) minus 0.12 times the BCODH activity. The latter term is necessary to correct for the contribution, at 1 mM-pyruvate, of BCODH to pyruvate decarboxylation. Therefore the expected 2-oxobutyrate decarboxylation rate equals 0.26 times the BCODH activity plus 0.43 times the PDH activity. The expected or calculated rate of oxidative decarboxylation of 2-oxobutyrate, obtained as above, was equal to the observed, and the ratio (expected/observed) was not different from 1 (0.98 ± 0.03 ; mean \pm S.E.M., $n = 15$) for all treatments. The expected rate of 2-oxobutyrate decarboxylation could not be determined in the presence of 3-fluoropyruvate, since the degrees of inhibition of 2-oxobutyrate and pyruvate decarboxylation were not equal (see above).

Extracts of kidney mitochondria had a PDH/BCODH

Table 2. Activities of oxidative decarboxylation of pyruvate, 3-methyl-2-oxobutyrate and 2-oxobutyrate in liver mitochondria

Treatments and assay conditions are as described in the Experimental section. All values are given as means \pm S.E.M. for the numbers of observations in parentheses, except with less than three determinations, where values for each determination are given. Oxidative-decarboxylation rates are given as munits/mg of protein. Calculations of expected rate of oxidative decarboxylation of 2-oxobutyrate are based on the oxidative decarboxylation of pyruvate and 3-methyl-2-oxobutyrate as given in the Results section. Abbreviation: N.D., not determined (see the Results section).

Substrate	Without phosphatase treatment			With phosphatase treatment			
	No addition	3-Fluoro-pyruvate	Antibody	No addition	3-Fluoro-pyruvate	Antibody	ATP
Pyruvate	13.8 \pm 0.5 (4)	5.4 \pm 2.6 (3)	10.0 \pm 0.4 (3)	25.3 \pm 1.3 (4)	14.7 (14.6, 14.8)	20.3 (16.4, 24.2)	5.4 (1.4, 9.5)
3-Methyl-2-oxobutyrate	40.0 \pm 3.0 (4)	37.3 \pm 2.5 (3)	6.7 \pm 1.4 (3)	38.2 \pm 2.2 (4)	37.0 (33.7, 40.4)	4.9 (3.8, 6.0)	17.6 (16.4, 18.9)
2-Oxobutyrate	17.8 \pm 0.6 (4)	16.1 \pm 2.2 (3)	5.8 \pm 0.3 (3)	20.4 \pm 0.8 (4)	14.0 (12.3, 15.8)	10.2 (7.9, 12.4)	8.3 (5.1, 11.5)
Expected with 2-oxobutyrate	16.4 \pm 0.6 (4)	N.D.	6.0 \pm 0.3 (3)	20.8 \pm 1.1 (4)	N.D.	10.0	7.0
Expected/observed with 2-oxobutyrate	0.93 \pm 0.05 (4)	N.D.	1.04 \pm 0.05 (3)	1.03 \pm 0.09 (4)	N.D.	0.99	0.87

activity ratio of slightly > 1 before phosphatase treatment and a ratio of 2 after phosphatase treatment. Total activities (munits/mg of protein) of PDH and BCODH were respectively approx. 38 and 18, with the percentages of active form being approx. 55 and 90%. The rate of decarboxylation of 2-oxobutyrate, regardless of treatment, remained less than that of the PDH activity but greater than that of BCODH activity. Antibody treatment inhibited 2-oxobutyrate decarboxylation more before phosphatase treatment (30%) than after (20%), whereas inhibition of the BCODH activity (95%) and PDH activity (5%) was the same regardless of phosphatase treatment. 3-Fluoropyruvate inhibited decarboxylation of 2-oxobutyrate (55%) more than PDH activity (35%), although BCODH activity was inhibited. Mitochondrial extracts (after phosphatase treatment) from heart and skeletal muscle had respectively approx. 15 and 40 times greater PDH activity (approx. 140 and 150 munits/mg of protein) than BCODH activity (approx. 9.3 and 3.5 munits/mg of protein). Antibody treatment did not substantially inhibit decarboxylation of 2-oxobutyrate or pyruvate, but did inhibit BCODH activity. 3-Fluoropyruvate inhibited decarboxylation of 2-oxobutyrate (75%) more than that of pyruvate (40%) in extracts of mitochondria from both tissues. The ratio of expected or calculated rate of 2-oxobutyrate decarboxylation to the observed rate for the mitochondrial extracts from all tissues under all treatments, except with 3-fluoropyruvate treatment, was 0.97 ± 0.03 (mean \pm S.E.M., $n = 61$).

Hydroxyapatite-chromatographic separation of two peaks of oxidative-decarboxylation activities towards 2-oxobutyrate

The predictability of the rate of 2-oxobutyrate decarboxylation (based on the activities of PDH and

BCODH, and kinetic constants), and the tissue differences in sensitivity to inhibition of 2-oxobutyrate decarboxylation by antibodies and 3-fluoropyruvate suggest that 2-oxobutyrate decarboxylation was either solely or predominantly due to the activities of PDH and BCODH. To investigate this possibility further, and the possibility of a 2-oxobutyrate dehydrogenase, an extract of liver mitochondria after phosphatase treatment was fractionated on hydroxyapatite (Fig. 4). This absorption matrix was shown to bind purified PDH and BCODH, the former being eluted before the latter (results not shown; also see Odessey, 1980). Two peaks which oxidatively decarboxylated 2-oxobutyrate were resolved, with the first and the second peaks being associated with PDH and BCODH activities respectively. The second peak also oxidatively decarboxylated pyruvate, which is consistent with the activity of BCODH towards pyruvate (see above). The oxidative decarboxylation of pyruvate and 2-oxobutyrate by peak 1 were not inhibited by antibody, whereas the activities with all three 2-oxo acids associated with peak 2 were inhibited (results not shown). A similar experiment with extracts of heart mitochondria, after phosphatase treatment, also revealed oxidative decarboxylation of 2-oxobutyrate associated with one peak of PDH activity and a later peak of BCODH activity (results not shown).

Competition studies between 2-oxo acids for oxidative decarboxylation in mitochondrial extracts

The large differences in apparent K_m values of 2-oxobutyrate with PDH and BCODH, the relative differences between apparent K_m values of both complexes for the different 2-oxo acids, the oxidative decarboxylation of pyruvate by both complexes, and the lack of oxidative decarboxylation of 3-methyl-2-oxobutyrate by PDH all suggest that the tissue-dependent variation in relative

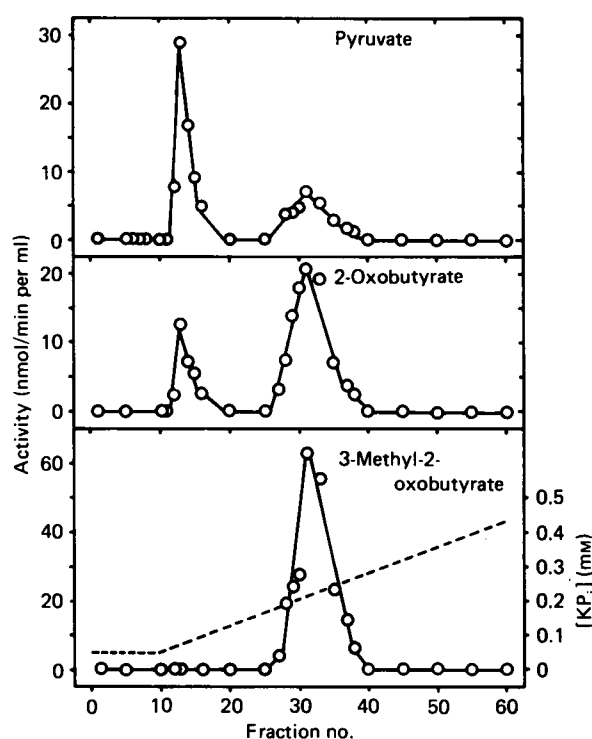


Fig. 4. Hydroxyapatite chromatography of liver mitochondrial extract

Extraction of liver mitochondria and treatment with phosphatase were as described in the Experimental section. The treated extract was centrifuged at 10000 *g* for 10 min in a Sorvall SS-34 rotor. Extract was made to 50 mM-potassium phosphate (pH 7.5), absorbed on hydroxyapatite (1 cm × 11 cm column), and the column was eluted with a 0.3 litre linear gradient of potassium phosphate (pH 7.5) from 50 to 500 mM (----). The spectrophotometric assay was as described in the Experimental section, with the activities (nmol/min per ml) shown for 1 mM substrate concentration with pyruvate (top), 2-oxobutyrate (middle) and 3-methyl-2-oxobutyrate (bottom).

activities of these complexes would influence the oxidative decarboxylation of a particular 2-oxo acid when in the presence of another. Competition studies with a 1-¹⁴C-labelled 2-oxo acid (1 mM) in the presence of various concentrations of unlabelled 2-oxo acids were

done with phosphatase-treated extracts of mitochondria from liver, heart and skeletal muscle (Table 3). Extracts of mitochondria from liver showed a quantitative difference in inhibition by the unlabelled 2-oxo acids compared with mitochondria from heart or skeletal muscle. Thus extracts of liver mitochondria were more sensitive (lower *I*₅₀ values) to inhibition of [1-¹⁴C]pyruvate decarboxylation by 3-methyl-2-oxobutyrate or 2-oxobutyrate, and to inhibition of 2-oxo[1-¹⁴C]butyrate decarboxylation by 3-methyl-2-oxobutyrate, than were extracts from heart or skeletal-muscle mitochondria. Conversely, liver mitochondrial extracts showed less sensitivity to inhibition of 2-oxo[1-¹⁴C]butyrate decarboxylation by pyruvate than did mitochondrial extracts from heart or skeletal muscle. These differences between liver and heart or skeletal-muscle mitochondria apparently reside in the relative activities of BCODH and PDH and their associated capacities to utilize these 2-oxo acids.

DISCUSSION

Several different approaches were used to evaluate the enzymes responsible for oxidative decarboxylation of 2-oxobutyrate. Kinetic analyses of the isolated complexes showed that 2-oxobutyrate was a better substrate with BCODH than with PDH, since the apparent *K*_m for 2-oxobutyrate was approx. 4-fold lower with BCODH (i.e. 0.03 rather than 0.12 mM) and there was only a small difference in apparent *K*_m values between 2-oxobutyrate (26 μM) and 3-methyl-2-oxobutyrate (35 μM). The phosphorylation state of both complexes in mitochondrial extracts was also shown to influence the rate of oxidative decarboxylation of 2-oxobutyrate. This suggests that only BCODH and PDH were responsible for oxidative decarboxylation of 2-oxobutyrate, since these are the only two intramitochondrial enzymes regulated by phosphorylation (Hughes & Halestrap, 1981). Antibodies to BCODH inhibited purified BCODH, BCODH activity in mitochondrial extracts from all tissues tested, and the decarboxylation of 2-oxobutyrate in mitochondria from liver and kidney. These antibodies, however, did not inhibit purified PDH or decarboxylation of pyruvate or 2-oxobutyrate in extracts from heart or skeletal muscle. Thus the antibodies inhibited BCODH and the concomitant oxidative decarboxylation of 2-oxo acid substrates of this complex, and probably not another enzyme, e.g. 2-oxobutyrate dehydrogenase. Decarboxylation of 2-

Table 3. Inhibition (*I*₅₀ values) of 1-¹⁴C-labelled 2-oxo acid decarboxylation by unlabelled 2-oxo acids in mitochondrial extracts

Mitochondrial extracts were treated with phosphatase. Each value represents two different determinations with at least five different concentrations of unlabelled 2-oxo acid with 1 mM 1-¹⁴C-labelled 2-oxo acid. *I*₅₀ is the concentration of unlabelled 2-oxo acid giving 50% inhibition of the decarboxylation of the 1-¹⁴C-labelled 2-oxo acid. Abbreviation: N.D., not determined.

Unlabelled 2-oxo acid	<i>I</i> ₅₀ values (mM) of indicated 2-oxo acid					
	[1- ¹⁴ C]Pyruvate			2-Oxo[1- ¹⁴ C]butyrate		
	Liver	Heart	Skeletal muscle	Liver	Heart	Skeletal muscle
3-Methyl-2-oxobutyrate	3.0	> 5	4.5	2.0	5	> 5
2-Oxobutyrate	0.75	1.5	1.6	N.D.	N.D.	N.D.
Pyruvate	N.D.	N.D.	N.D.	1.4	0.25	0.3

oxobutyrates was inhibited more by antibody treatment in liver mitochondria and more by 3-fluoropyruvate in heart and skeletal-muscle mitochondria. Kidney mitochondria showed a response intermediate between these tissues' mitochondria. This pattern of inhibition apparently reflects relative amounts of these complexes in mitochondria, since the total PDH and BCODH activity ratio is slightly < 1 in liver, > 2 in kidney, > 15 in heart and > 40 in skeletal muscle. The rate of oxidative decarboxylation of 2-oxobutyrates could also be calculated from the activities of PDH and BCODH, and the kinetic constants obtained with the purified complexes. This calculated rate was the same as that observed, regardless of treatment with phosphatase, ATP or antibody. Also, only two peaks of 2-oxobutyrates oxidative-decarboxylation activity from liver and rat heart mitochondria extracts were separated on hydroxyapatite, one associated with PDH and the other with BCODH activity. These data strongly suggest that the combined activities of these complexes were solely responsible for the oxidative decarboxylation of 2-oxobutyrates.

BCODH has been previously reported to catalyse oxidative decarboxylation of pyruvate (Pettit *et al.*, 1978; Paxton & Harris, 1982), although this has not generally been considered in the assay of PDH, or in competition studies with mitochondria involving pyruvate and the branched-chain 2-oxo acids. This overestimation of PDH activity would only be apparent in tissues (i.e. liver and kidney) where BCODH represents a substantial activity relative to PDH and would become even more apparent with high pyruvate concentrations, owing to the high apparent K_m for pyruvate of the BCODH. For example, in liver mitochondrial extracts before phosphatase treatment, PDH activity with 1 mM-pyruvate was calculated to be overestimated by approx. 4.8 munits/mg of protein, or 50%, whereas with 5 mM-pyruvate the calculated overestimation owing to BCODH activity would apparently have been 7.2 munits/mg of protein, or 75% (based on kinetic data). This contribution of BCODH to pyruvate decarboxylation was obvious in the present study, as antibodies to this complex decreased pyruvate decarboxylation in liver mitochondria by the amount calculated (based on the kinetic data) to be contributed by this complex. Also, 3-methyl-2-oxobutyrates was a more potent inhibitor of pyruvate decarboxylation in liver mitochondria (I_{50} approx. 3 mM) than in heart or skeletal muscle mitochondria (I_{50} approx. 5 mM). It is not clear whether BCODH oxidatively decarboxylates pyruvate *in vivo*; however, there is interest (see Gaull *et al.*, 1975) in the inhibition of pyruvate decarboxylation by branched-chain 2-oxo acids as a possible mechanism for some of the abnormalities associated with maple-syrup-urine disease. This disease is characterized by elevated concentrations of branched-chain 2-oxo acids and a genetically defective BCODH (see Dancis & Levitz, 1978). Several studies with liver mitochondria (Bowden *et al.*, 1970; Johnson & Connelly, 1972; McArthur & Bowden, 1972) and kidney slices or homogenates (Dawson & Hird, 1968) have shown inhibition of pyruvate decarboxylation by branched-chain 2-oxo acids, whereas isolated PDH is not very sensitive to inhibition by branched-chain 2-oxo acids (Blass & Lewis, 1973). The inhibition of pyruvate decarboxylation by branched-chain 2-oxo acids may reflect the relative differences in apparent K_m values between pyruvate and the 2-oxo acids with BCODH, and not inhibition of PDH.

Thus the inhibition of PDH activity or pyruvate decarboxylation by elevated concentrations of branched-chain 2-oxo acids may be exaggerated, and the influence of branched-chain 2-oxo acids on pyruvate metabolism needs further study.

Competition studies showed that liver mitochondria gave a pattern of inhibition different from those of heart or skeletal muscle as also observed by Bremer (1969) with intact mitochondria. For example, in mitochondria from heart or skeletal muscle, where the total PDH/BCODH total-activity ratio was > 15 , 2-oxobutyrates decarboxylation was more sensitive to pyruvate inhibition (I_{50} approx. 0.25 mM) than was inhibition of pyruvate decarboxylation by 2-oxobutyrates (I_{50} approx. 1.5 mM). This reflects, presumably, the higher apparent K_m for 2-oxobutyrates relative to pyruvate with PDH. If the intracellular concentration of pyruvate is greater than that of 2-oxobutyrates, as suggested by various determinations (Koike & Koike, 1984; Walajtys-Rode *et al.*, 1984), then the decarboxylation of 2-oxobutyrates by PDH will be very limited. However, with liver mitochondria, where the PDH/BCODH total-activity ratio was < 1 , 2-oxobutyrates decarboxylation was less sensitive to pyruvate inhibition (I_{50} approx. 1.4 mM) than was pyruvate decarboxylation by 2-oxobutyrates (I_{50} approx. 0.75 mM). This reflects the contribution of BCODH and its concomitantly higher apparent K_m for pyruvate relative to 2-oxobutyrates. The contribution of hepatic BCODH to 2-oxobutyrates decarboxylation would be even greater *in vivo*, since BCODH is $> 90\%$ active (Gillim *et al.*, 1983) and PDH $< 10\%$ active (Wieland *et al.*, 1972) with the activity ratio PDA/BCODH of < 0.1 . Thus the differences in K_m values for the 2-oxo acids between PDH and BCODH, as reflected in the competition studies, and the apparent physiological concentrations of the 2-oxo acids (see above and Hutson & Harper, 1981) suggest that BCODH, particularly in liver, would be predominantly responsible for 2-oxobutyrates decarboxylation.

Liver is normally responsible for the catabolism of threonine and methionine (see the Introduction section) and also contains, normally, the greatest activity of BCODH relative to other tissues (Gillim *et al.*, 1983; Patston *et al.*, 1984). The percentage of active form and total BCODH activity in liver can be altered by various conditions. For example, the total activity and percentage of active form of this complex in liver is low in rats fed on a low-protein diet, and both become greater with higher-protein diets (Gillim *et al.*, 1983; Harris *et al.*, 1985). Rats fed on diets with proportionally greater protein content also show increased hepatic 2-oxobutyrates decarboxylation (Steele *et al.*, 1984) and increased catabolism of methionine and threonine (see Harper & Benjamin, 1984). The increased catabolism of methionine and threonine may, however, only partially reflect the changes in BCODH activity, as there are other changes in the enzymes associated with the catabolic pathways of these amino acids (Finkelstein & Martin, 1984; Peters & Harper, 1985). Further evidence of the primary role of this complex in 2-oxobutyrates catabolism is seen in maple-syrup-urine disease. The urine of these patients contains 2-hydroxybutyrates (Jakobs *et al.*, 1977), an excretion metabolite of 2-oxobutyrates. 2-Hydroxybutyrates or a polymer of this compound also gives the urine of these patients the characteristic odour (Menkes, 1959). Thus this inborn error of metabolism also appears to involve a defect in the normal oxidative decarboxylation

of 2-oxobutyrate, presumably owing to a defective BCODH.

Extrahepatic tissues, with the possible exception of kidney, normally contain a very low percentage of active form of BCODH (Gillim *et al.*, 1983; and Patston *et al.*, 1984) and consequently do not normally contribute substantially to the oxidative decarboxylation of the branched-chain 2-oxo acids. Several compounds, however, can inhibit isolated BCODH kinase and also activate this complex and increase the flux through this complex in perfused rat hearts (Paxton & Harris, 1982, 1984a,b). These compounds therefore may increase the role of extrahepatic tissues in branched-chain 2-oxo acid and 2-oxobutyrate catabolism via activation of this complex. Phenylpyruvate, which accumulates in the blood and tissues of phenylketonuric patients owing to a defect in phenylalanine hydroxylase (Tourian & Sidbury, 1978), was also part of this group of BCODH kinase inhibitors. Patients with this inborn error of metabolism show lower concentrations of branched-chain amino acids, methionine, threonine and 2-aminobutyrate (Elfron *et al.*, 1969). This may be due to activation of BCODH in extrahepatic tissues. Clofibrate acid, a hyperlipidaemic agent and an inhibitor of BCODH kinase (Paxton & Harris, 1984b), lowers blood concentrations of branched-chain amino acids and threonine (Wolfe *et al.*, 1973). The branched-chain 2-oxo acids also inhibit BCODH kinase (Paxton & Harris, 1982, 1984a) and may partially explain the influence of dietary protein contents on increased threonine and methionine metabolism (see above), since increasing dietary protein leads to increased blood concentrations of the branched-chain 2-oxo acids (Hutson & Harper, 1981). Chemically induced diabetes in rats also results in elevated blood concentrations of branched-chain 2-oxo acids (Hutson & Harper, 1981) and 2-aminobutyrate (Brosnan *et al.*, 1983), suggesting, on the basis of data in the present paper, a common mechanism in the catabolism of these 2-oxo acids.

In conclusion, 2-oxobutyrate is oxidatively decarboxylated primarily if not solely by BCODH and PDH. No evidence was seen of a specific 2-oxobutyrate dehydrogenase, and the rate of oxidative decarboxylation of 2-oxobutyrate was predictable on the basis of the combined activities of BCODH and PDH. It would appear that BCODH plays a greater role in oxidative decarboxylation of 2-oxobutyrate than does PDH, on the basis of kinetic analyses of the isolated complexes, competition studies with extracts of mitochondria, and apparent physiological concentrations of pyruvate, 2-oxobutyrate and the branched-chain 2-oxo acids. This may partially explain why liver, which normally has more active and total BCODH than other tissues, is primarily responsible for the catabolism of threonine and methionine. Since both complexes are regulated by phosphorylation, then those factors that influence the phosphorylation state of these complexes with a subsequent influence on the catabolism of pyruvate and the branched-chain amino and 2-oxo acids will also influence the catabolism of 2-oxobutyrate. BCODH has now been shown to be directly involved with the oxidative decarboxylation of the 2-oxo acids corresponding to at least five essential amino acids: leucine, isoleucine, valine, threonine and methionine.

We thank Dr. Arthur Schulz for helpful discussions, Bonnie McGarr-Bousani for technical assistance, Peggy Smith for

expert typing of the manuscript, and financial support from NIH Grants AM19259 and AM13939, American Cancer Society Institutional Grant IN-161, the Indiana Affiliate of American Diabetes Association, and the Grace M. Showalter Residuary Trust.

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Received 5 August 1985/14 October 1985; accepted 29 October 1985