BRANCHED CHAIN 2-OXO-ACID DEHYDROGENASE COMPLEX OF RAT LIVER

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1. Introduction

It is generally assumed that branched chain 2-oxo-acids formed from leucine, isoleucine and valine are oxidised by dehydrogenase complex(es) analogous to pyruvate and 2-oxoglutarate dehydrogenase complexes. There is much recent work on the regulation of oxidation of branched chain amino acids and 2-oxo-acids in vivo but little information on substrate kinetics or regulation by metabolite effectors of the branched chain 2-oxo-acid dehydrogenase complex(es). We describe here a method for extraction and partial purification of a complex from rat liver mitochondria, K_m values for substrates and inhibition by isovaleryl CoA (competitive with CoA) and NADH (competitive with NAD^+). Evidence is given that a single complex may oxidise all 3 branched chain 2-oxo-acids.

2. Experimental

2.1. *Materials*

Sodium salts of OIC, OIV and OMV were from Sigma (London) Chemical Co., Kingston-upon-Thames; other biochemicals were from Boehringer Corp. (London) Ltd., London, W5 UK; chemicals were from British Drug Houses Ltd., Poole, Dorset UK. Isovaleryl CoA (free of CoA) was synthesized in 86% yield from isovaleryl anhydride [1] and assayed spectrophotometrically with carnitine acetyltransferase [2]. Solutions of branched chain 2-oxo-acids were standardised spectrophotometrically with the branched chain 2-oxo-acid dehydrogenase (with 1 mM L-carnitine and 2 units of carnitine acetyl transferase to remove branched chain acyl CoA).

Branched chain 2-oxo-acid dehydrogenase complex was partially purified as follows. The mitochondrial pellets from 25 rat livers [3] were freed of lysosomes by incubation at 0°C for 2 min in 0.25 M sucrose/5 mM Tris–HCl/2 mM EDTA/1 mg/ml digitonin/pH 7.5 (20 ml/liver). The mitochondrial suspension was then diluted with sucrose/Tris/EDTA (80 ml/liver), mitochondria separated (30 min, 23 000 × g, 2°C) and washed twice by resuspension in sucrose/Tris/EDTA (180 ml/liver) and centrifugation. The pellets were suspended in 30 mM potassium phosphate/3 mM EDTA/3 mM 2-mercaptoethanol/5% (v/v) Triton X-100/pH 7.5 and frozen (liquid N_2) and thawed × 3. The supernatant (45 min, 54 000 × g, 2°C) was centrifuged (150 min, 180 000 × g, 2°C), the pellets taken up in 30 mM potassium phosphate/3 mM DTT/pH 7, the pH adjusted to 7.3 with 0.1 M KOH and 0.08 vol. 50% (w/v) aqueous polyethylene glycol added with stirring. The supernatant (5 min, 38 000 × g, 2°C) was adjusted to pH 6.8 with 0.1 M acetic acid and the pellet (10 min, 38 000 × g, 2°C) taken up in 3.2 ml phosphate/DTT. This first fraction of branched chain 2-oxo-acid dehydrogenase complex (3.2 units with OIC; 0.12 units/mg protein) was free of pyruvate and 2-oxoglutarate dehydrogenases. A further fraction was precipitated from the pH 6.8 supernatant at pH 6.5; this second fraction (6 units; 0.24 units/mg protein) contained pyruvate and 2-oxoglutarate dehydrogenase complexes (0.6 and 1.1 unit, respectively). The total yield in these 2 fractions was 10% of that extracted with Triton X-100.

Abbreviations: TPP, thiamin pyrophosphate; DTT, dithiothreitol; OIC, 4-methyl-2-oxopentanoate (2-oxoisocaproate); OIV, 3-methyl-2-oxobutyrate (2-oxoisovalerate); OMV, DL-3-methyl-2-oxopentanoate (DL-2-oxo-3-methylvalerate)

2.2. *Assays and calculations*

Branched chain 2-oxo-acid dehydrogenase complex activity was assayed spectrophotometrically at 30°C by the initial rate of $\Delta E_{340\,nm}$ on adding OIC, OIV or OMV to 30 mM potassium phosphate/1.8 mM $MgSO_4$/2 μg rotenone/pH 7.5 containing enzyme (5–20 milliunits in 5–20 μl), CoA, TPP and NAD^+ in vol. 0.5 ml (for other concentrations see table 1 or fig.1). Both fractions of dehydrogenase showed low NADH oxidase activity (0.07 units/ml) inhibited by rotenone; in mitochondrial extracts NADH oxidase was inhibited with 1 mM KCN. Pyruvate and 2-oxoglutarate dehydrogenase complexes were assayed as in [4,5]. One unit of enzyme activity is defined by the formation of 1 μmol product/min at 30°C with substrate concentrations of not less than 20 × app. K_m.

Apparent K_m, V_{max} and K_i were computed [6–8]. In determining apparent K_m and V_{max} values the concentrations of other substrates and co-factors were at least 10-fold greater than their app. K_m. The 2 fractions of branched chain 2-oxo-acid dehydrogenase complex were only partially dependent (0.75) on added TPP. It was assumed that the rate in the absence of added TPP is due to TPP in the preparation and the app. K_m for TPP was calculated from the slope in least squares linear regression analysis of S against $[(V_m/v_s-1)^{-1} - (V_m/v_o-1)^{-1}]$ where S is concentration of added TPP; V_m is initial velocity with 33.3 mM TPP; v_s is initial velocity at added TPP concentration S; and v_o is initial velocity in absence of added TPP.

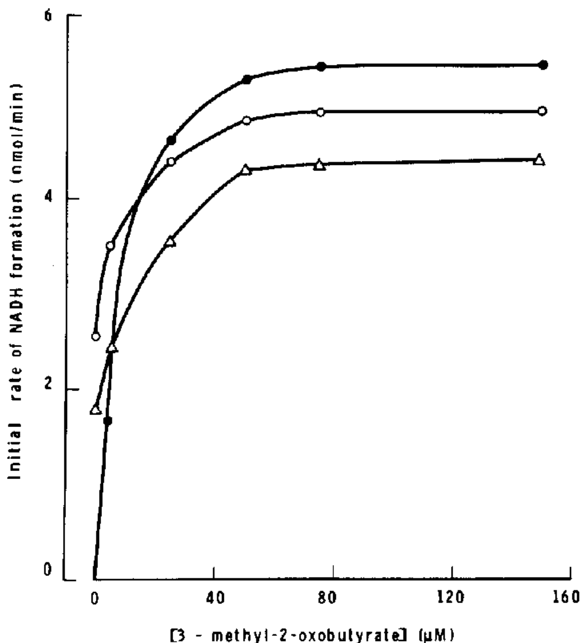

Fig.1. Initial rates were measured spectrophotometrically at 30°C by the ΔE_{340} nm, the reaction being initiated with 2-oxo-acid(s). Assay buffer was 30 mM potassium phosphate/1.8 mM $MgSO_4$/pH 7.5. Other concentrations were: 0.4 mM CoA; 0.4 mM thiamin pyrophosphate; 1 mM NAD; (●——●) no other addition; (○——○) 20 μM 4-methyl-2-oxopentanoate; (△——△) 20 μM DL-3-methyl-2-oxopentanoate.

3. Results and discussion

With either fraction of branched chain 2-oxo-acid dehydrogenase complex, reduction of NAD^+ was absolutely dependent on CoA and 2-oxo-acid and partially (0.75) dependent on TPP. Addition of Mg^{2+} was not required but dependence on Mg^{2+} was suggested by inhibition (75%) with 5 mM EDTA reversed by 5 mM $MgSO_4$. As described in section 2.1, the complex in 5% Triton extracts of mitochondria sedimented after 150 min at 180 000 × *g* from which it is inferred that it is of a high molecular weight comparable to that of the pyruvate and 2-oxoglutarate dehydrogenase complexes (10^6 – 10^7 daltons).

Apparent K_m values for CoA, TPP, OIC, OIV and OMV are given in the table and there was no significant difference for the values obtained with each of the three 2-oxo-acids used. The app. K_m value for OMV is complex as the DL form of this 2-oxo-acid was used. Recovery experiments in which the dehydrogenase reaction was taken to completion (see section 2.1) were consistent with oxidation of both D and L forms according to the equation (3-methyl-2-oxopentanoate + CoA + NAD → 2-methyl-butyryl CoA + $NADH_2$ + CO_2). With each of the three 2-oxo-acids, the dehydrogenase reaction was inhibited by isovaleryl CoA (competitive with CoA) and by NADH (competitive with NAD^+). The K_i values did not vary significantly with each of the three 2-oxo-acids. The V_{max} values differed significantly in the order OIV > OIC > OMV. The K_m values for 2-oxo-acids and the relative V_{max} values only were obtained with a preparation of branched chain 2-oxo-acid dehydrogenase which contained pyruvate and 2-oxoglutarate

Table 1
Kinetic constants for rat liver branched chain 2-oxo-acid dehydrogenase complex

Experiments	Mean ± SEM		
	4-methyl-2-oxopentanoate (OIC)	3-methyl-2-oxobutyrate (OIV)	DL-3-methyl-2-oxopentanoate (OMV)
1. K_m for 2-oxo-acid substrate (μM)	14.6 ± 1.7	13.0 ± 3.2	10.5 ± 1.5
Relative V_{max} (nmol NAD formed/min)	18.2 ± 0.52	23.4 ± 1.4	11.7 ± 0.4
2. K_m for NAD^+ (μM)	42.3 ± 1.9	39.5 ± 3.1	38.7 ± 1.2
K_i for NADH (μM)	18.0 ± 1.0	17.7 ± 4.5	23.0 ± 2.9
3. K_m for CoA (μM)	10.1 ± 1.2	10.6 ± 1.1	7.5 ± 0.7
K_i for isovaleryl CoA (μM)	13.6 ± 2.1	8.2 ± 1.0	10.1 ± 1.1
4. K_m for thiamin pyrophosphate (μM)	–	1.2 ± 0.14	–

K_m values are app. K_m. For general details of assay and calculations see section 2.2. Fixed concentrations as appropriate were 0.4 mM TPP; 0.4 mM CoA (0.18 mM in expt. 2); 1 mM NAD^+; 0.15 mM 2-oxo-acids. Expt. 1: at least 15 2-oxo-acid concentrations, 10–200 μM. Expt. 2: 8 NAD^+ concentrations, 25–620 μM; at 0 μM and 8.4 μM NADH (all three 2-oxo-acids); at 42 μM and 174 μM NADH (OIC only). Expt. 3: 8 CoA concentrations, 5.5–425 μM; at 0 μM, 16.6 μM, 33.2 μM and 82.8 μM isovaleryl CoA. Expt. 4: 15 concentrations added TPP 60 nM – 33.3 μM

dehydrogenases. Experiments with purified pig heart pyruvate and 2-oxoglutarate dehydrogenase complexes have shown no activity towards the branched chain 2-oxo-acids.

As shown in fig.1 there was no evidence for additive rates of NAD^+ reduction comparing OIV alone (5–150 μM) or OIV plus 20 μM OIC or OIV plus 20 μM OMV. This may suggest that a single complex oxidises all 3 branched chain 2-oxo-acids. This is consistent with conclusions based on the decarboxylation of branched chain 2-oxo-acids by washed rat liver mitochondria [9]. Our preparations differ from a preparation from ox-liver which suggested the presence of branched chain 2-oxo-acid dehydrogenase complexes with app. K_m values for 2-oxo-acids in the mM range [10].

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