INACTIVATION OF RAT LIVER AND KIDNEY BRANCHED CHAIN 2-OXOACID DEHYDROGENASE COMPLEX BY ADENOSINE TRIPHOSPHATE

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Received 9 February 1981

1. Introduction

Rat heart and skeletal muscle branched-chain 2-oxoacid dehydrogenase (BCDH) complex is rapidly inactivated by ATP [1–6]; inactivation by ATP may result from phosphorylation [2,5,7] of the α-subunit of BCDH [8]. Inactivation and phosphorylation are inhibited by ketoleucine [1,2,5,6]. Reactivation of inactive BCDH complexes may be effected with partially purified preparations of pyruvate dehydrogenase phosphate phosphatase [6]. In rat heart and in freshly prepared rat heart or skeletal muscle mitochondria 85–95% of BCDH complex is in the inactive form [2,6]. Active complex is formed in mitochondria incubated with ketoleucine or depleted of ATP by incubation without respiratory substrate or by incubation with uncouplers; in mitochondria incubated with respiratory substrates 90–95% of complex is in the inactive form [2,6]. Active complex is formed in rat hearts perfused with medium containing ketoleucine or leucine [6].

Only active BCDH complex has been detected in freshly prepared rat liver or kidney mitochondria [4,6]. Inactivation of BCDH complex by ATP was not detected although inactivation of the pyruvate dehydrogenase complex by ATP was detected in the same experiment [6]. This led to the suggestion of tissue-specific differences in the interconversion of active and inactive BCDH complexes between muscles (on the one hand) and liver and kidney [6]. This could reflect, for example, for different isoenzymes of BCDH or absence or inhibition of the inactivating enzyme (presumed to be a protein kinase) in liver and kidney. It is shown here that BCDH complex in extracts of rat liver mitochondria is inactivated by ATP after a lag period but at a slower rate than the heart muscle complex. Inactivation of kidney complex is also shown; it was also slower and less extensive than that of heart muscle. Inactivation of liver and kidney complexes was inhibited by branched-chain 2-oxoacids and evidence is given that inactivation may be associated with phosphorylation of the α-chain of BCDH. Evidence for ATP-induced phosphorylation and inactivation of BCDH complex in rat kidney mitochondria has appeared in [9].

Abbreviations: BCDH, branched-chain 2-oxoacid dehydrogenase; EGTA, ethanedioxybis(ethylamine)-tetraacetate; TLCK, *N*-α-*p*-tosyl-L-lysine chloromethyl ketone; DTT, dithiothreitol; ketoleucine, 4-methyl-2-oxopentanoate; ketoisoleucine, D,L-3-methyl-2-oxopentanoate; ketovaline, 3-methyl-2-oxobutyrate; TPP, thiamin pyrophosphate; SDS, sodium dodecyl sulphate; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone

2. Experimental

Sources of biochemicals and chemicals are given in [1,6]. [γ-^{32}P]ATP was from the Radiochemical Centre, Amersham. Rat heart, liver and kidney mitochondria were prepared as in [6]. Mitochondria were incubated (2.5 mg protein in 1 ml), separated and frozen as in [6]. The period of incubation was 30 min; aeration of medium was renewed at 6–8 min intervals by 1 s of vortex mixing. Additions to incubation medium are given in section 3. Extracts for assay of BCDH complex were prepared by ultrasonic disintegration of the frozen pellet into 250 μl extraction buffer (30 mM potassium phosphate/2 mM EDTA/5 mM DTT/1 mM TLCK/2% (v/v) ox-serum (pH 7.5). BCDH complex was assayed in 100 μl extract. For ATP inactivation extracts were prepared as in [1] from frozen pellets of freshly prepared liver or kidney mitochondria; or heart mitochondria incubated for 15 min

without respiratory substrate [1]. The extraction buffer (see above) contained 10 mM EGTA in place of 2 mM EDTA and oligomycin B, 25 μg/ml. Extracts (5.3 mg mitochondrial protein/ml) were warmed to 30°C (5 min) and reaction initiated with 0.05 vol. ATP^+-branched chain 2-oxoacids (in controls buffer replaced ATP). BCDH complex was assayed on 140 μl incubate. Incorporation of ^{32}P from [γ-^{32}P]ATP (1500 dpm/pmol) into BCDH used the same buffer (100 μl) with addition of 1 mM dichloroacetate to inhibit pyruvate dehydrogenase phosphorylation: dichloroacetate does not inhibit BCDH phosphorylation or inactivation in mitochondrial extracts [2,5]. Reaction was terminated with trichloroacetic acid to 10% (v/v), precipitated protein washed X6 with 1 ml 10% trichloroacetic acid, dissolved in 250 μl 50 mM Tris–HCl (pH 6.8) containing 1% (w/v) SDS, 150 mM 2-mercaptoethanol, 10% (v/v) glycerol, 125 μg/ml bromophenol blue and neutralised (6 M NaOH). Samples (15 μl) were subjected to SDS–polyacrylamide gel electrophoresis in 8 X 7 X 0.2 cm slabs (10% polyacrylamide) at 70–75 V. Gel were prepared, and the running buffer (Tris–glycine) was as in [10]. Autoradiographs of gels dried in vacuo were prepared on Kodak Blue Brand BB5 X ray film.

BCDH complex was asseyed spectrophotometrically by the initial rate of NAD^+ reduction followed at 30°C at 340 nm. The assay buffer (final vol. 1.4 ml) was 30 mM potassium phosphate/2 mM $MgSO_4$/2 mM KCN/0.4 mM TPP/0.4 mM CoA/1 mM NAD^+/0.2 mM ketoleucine/10 units lipoamide dehydrogenase/0.1% (v/v) Triton X-100 (pH 7.5). Extracts containing ketoisoleucine or ketovaline were assayed with the corresponding 2-oxoacid in place of ketoleucine. Progress curves were linear for at least 3 min (Gilford recording spectrophotometer; full scale extinction 0.1). There was no detectable NADH oxidase activity in extracts under the conditions of assay. The blank rate (measured in the absence of branched-chain 2-oxoacid) was negligible except for mitochondria incubated with glutamate (blank rate ~ 40% or with 2-oxoglutarate + L-malate (blank rate ~ 20%). These blank rates (which were subtracted) presumably reflect activities of glutamate, 2-oxoglutarate and malate dehydrogenases which are expressed when mitochondrial extracts contain their substrates. One unit of BCDH complex formed 1 μmol NADH/min at 30°C.

3. Results and discussion

3.1. *Inactivation of BCDH complex by ATP in extracts of rat liver and kidney mitochondria*

In these experiments as in [1], Mg^{2+} for ATP inactivation was derived from mitochondria; the computed concentration was ~160 μM. Further Mg^{2+} was not added because Mg^{2+} activates conversion of inactive BCDH complex into active complex [6]. The ATPase activity of mitochondrial extracts was limited by oligomycin B and restricting mitochondrial protein concentration. Measured ATPase activities by the method in [11] (liver first) as % of 0.5 mM ATP were, 12,23 in 5 min, 26,36 in 10 min, 47,53 in 20 min and 63,69 in 30 min (means of duplicate).

As shown in fig.1 BCDH complex in extracts of liver mitochondria was inactivated by 0.5 mM ATP; inactivation was inhibited completely by 1.4 mM

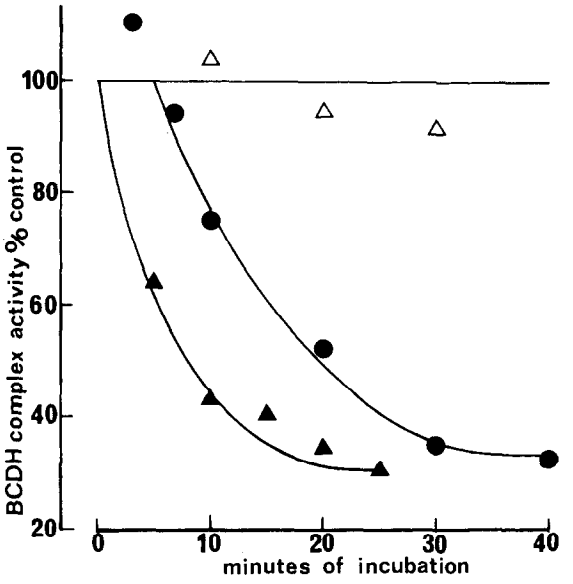

Fig.1. Inactivation of branched-chain 2-oxoacid dehydrogenase complex in extracts of rat liver mitochondria by ATP. Extracts of rat liver mitochondria were incubated with or without 0.5 mM ATP and assayed for branched-chain 2-oxoacid dehydrogenase complex at times shown. Activities with ATP are shown as % of activity in control (incubation without ATP): (●) 0.5 mM ATP added at zero time; (△) 0.5 mM ATP + 1.4 mM ketoleucine added at zero time; (▲) 0.5 mM ATP added after 15 min preincubation. Each point is mean of 8 obs. The initial activity of BCDH complex was 6.01 units/g mitochondrial protein. $P < 0.01$ for difference from 100% for all (▲); and for all (●) at or after 10 min; $P > 0.05$ for all other points.

ketoleucine. Inactivation by ATP only occurred after a lag period and significant ATP inactivation was first detected after 10 min incubation. This lag period was abolished by preincubation of mitochondrial extract for 15 min at 30°C prior to addition of ATP (fig.1). Inactivation by ATP was slow; ~70% of complex was inactivated in 30 min (no preincubation) or 20 min (preincubation). Inactivation by ATP was much slower than in heart mitochondrial extracts in which complete inactivation is achieved in 10 min (see [1] or table 1). In the experiments shown in fig.1 activity of BCDH complex (with ATP) is expressed as a percentage of the control (incubated under otherwise identical conditions without ATP). The initial activity of BCDH complex in liver mitochondrial extracts was 6.02 ± 0.06 (mean ± SEM) in units/g mitochondrial protein for 33 obs. on 4 mitochondrial preparations). Activity of BCDH complex in extracts incubated without ATP was stable and may have increased slightly. Activities as % of zero time were 98 ± 1.4 (3 min), 106 ± 3 (6 min), 115 ± 3 (10 min) 119 ± 1 (20 min) and 111 ± 1.3 (30 min) (mean ± SEM) for 8 obs.).

Ketoleucine inhibited inactivation of BCDH complex in extracts of rat liver mitochondria (table 1); 50% inhibition was achieved at 22–43 μM. Inhibition by ketoisoleucine was comparable to that of ketoleucine; inhibition by ketovaline was much less marked (table 1). In heart mitochondrial extracts (included for comparison) 50% inhibition by ketoleucine was achieved between 87 and 175 μM ketoleucine. Relative activities of liver BCDH complex with the three 2-oxoacids (line 1, table 1) were comparable to those in [12].

BCDH complex was inactivated by ATP in extracts of rat kidney mitochondria; activities (% of control lacking ATP) were 62% (5 min), 45% (10 min), 31% (20 min) and 27% (30 min) (mean of duplicates). Inactivation by 0.5 mM ATP was inhibited by 1 mM ketoleucine; activities after 30 min were (mean ± SEM; 4 obs.; units/g mitochondrial protein) 4.6 ± 0.09 (no ATP), 0.7 ± 0.09 (ATP). 4.1 ± 0.04 (ATP + ketoleucine) ($P < 0.001$ for effects of ATP or ketoleucine).

3.2. *Incorporation of ^{32}P from [γ-^{32}P]ATP*

SDS–polyacrylamide gel electrophoresis in Tris–glycine of heart mitochondrial extracts incubated with [γ-^{32}P]ATP separates two bands of ^{32}P corresponding in mobility to the α-chain of pyruvate

Table 1
Effect of branched-chain 2-oxoacids on inactivation of rat liver and rat heart branched-chain 2-oxoacid dehydrogenase complex by ATP

Ketoacid (μM)	Branched-chain 2-oxoacid dehydrogenase complex activity (mean ± SEM in units/g mitochondrial protein)			
	Heart	Liver		
	Ketoleucine	Ketoleucine	Ketoisoleucine	Ketovaline
0 (zero ATP)	2.75 ± 0.04[b] (8)	6.1 ± 0.14[b] (8)	3.2 ± 0.23[b] (4)	7.2 ± 0.12[b] (4)
0	−0.20 ± 0.12[a] (7)	2.1 ± 0.03[a] (8)	1.5 ± 0.05[a] (4)	1.9 ± 0.03[a] (4)
1400	2.91 ± 0.09[b] (4)	5.9 ± 0.47[b] (12)	3.3 ± 0.06[b] (4)	4.8 ± 0.17[a,b] (4)
700	2.55 ± 0.09[b] (3)	6.5 ± 0.98[b] (4)	–	–
350	2.00 ± 0.01[a,b] (4)	5.9 ± 0.11[b] (4)	2.6 ± 0.23[b] (4)	2.0 ± 0.03[a] (4)
175	1.69 ± 0.03[a,b] (3)	–	–	–
87	0.80 ± 0.03[a,b] (3)	–	–	–
43	0.88 ± 0.06[a,b] (3)	5.3 ± 0.11[a,b] (7)	2.1 ± 0.12[a] (4)	–
22	–	2.6 ± 0.05[a,b] (7)	–	–
11	–	2.0 ± 0.06[a,b] (8)	–	–

[a] $P < 0.01$ against zero ATP; [b] $P < 0.01$ against zero ketoacid; no obs. in parentheses

Mitochondrial extracts (5 mg protein/ml) were incubated at 30°C with 0.5 mM ATP (except line 1, zero ATP) and ketoacid at μM shown for 10 min (heart) or 20 min (liver). Branched-chain 2-oxoacid dehydrogenase complex activity was then assayed spectrophotometrically (NAD + reduction by ΔE_{340}), employing the ketoacid shown at the head of each column. For further details of methods used see section 2

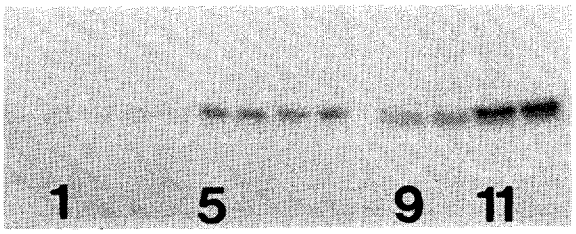

Fig.2. Autoradiograph of SDS–polyacrylamide gel electrophoresis in Tris–glycine buffer of rat liver and rat kidney mitochondrial extracts incubated with 0.5 mM [γ-^{32}P]ATP (1500 dpm/pmol) ± 2 mM ketoleucine for 30 min at 30°C. Proteins were precipitated and washed (×6 with 10% trichloroacetic acid, taken up in SDS and neutralised and subjected to electrophoresis in 10% polyacrylamide. Tracks are (left–right): (1–4) liver, 0.5 mM ATP + 2 mM ketoleucine; (5–8) liver, 0.5 mM ATP; (9,10) kidney, 0.5 mM ATP + 2 mM ketoleucine; (11,12) kidney, 0.5 mM ATP. For further details see section 2. The origin is at the top.

dehydrogenase ($M_r \sim 43\,000$) and the α chain of BCDH ($M_r \sim 48\,000$). Incorporation into the lower M_r band is inhibited by pyruvate and dichloroacetate; incorporation into the higher M_r band is inhibited by ketoleucine [5]. Comparable data have been obtained with extracts of rat liver and kidney BCDH incubated with [γ-^{32}P]ATP, dichloroacetate ± ketoleucine (fig.2). Autoradiographs showed 2 bands of radioactivity. The lower M_r ^{32}P band was weak and poorly reproduced in fig.2 because extracts of freshly prepared rat liver or kidney mitochondria contain only ~10% of active pyruvate dehydrogenase complex; and ^{32}P incorporation into the pyruvate dehydrogenase complex was inhibited by 1 mM dichloroacetate. Incorporation of ^{32}P into the higher M_r band was markedly inhibited by 1 mM ketoleucine. Ketoleucine is only a weak inhibitor of pyruvate dehydrogenase phosphorylation [11]. Inactivation of rat liver and kidney BCDH complex by ATP may thus be correlated with phosphorylation of the α-subunit of BCDH. Incorporation of ^{32}P from [γ-^{32}P]ATP into the α-subunit in BCDH in intact rat liver [8] and kidney [9] mitochondria has been suggested.

3.3. *BCDH activity in rat liver and kidney mitochondria*

Because inactivation of BCDH complex by ATP in extracts of rat liver mitochondria and (to a lesser extent) rat kidney mitochondria was slow we have reinvestigated effects of respiratory substrates, uncouplers and ketoleucine on BCDH complex activity in intact mitochondria with more prolonged incubation (30 min). No change in activity followed incubation with 5 mM glutamate ± 1.4 mM ketoleucine (initial activity 5.6 ± 0.06, 13 obs.; glutamate 5.6 ± 0.12, 4 obs.; glutamate + ketoleucine 5.6 ± 0.19, 4 obs.; mean ± SEM in units/g mitochondrial protein). A small decrease to 4.8 ± 0.08 was observed with 5 mM 2-oxoglutarate + 0.5 mM L-malate (mean ± SEM; 13 obs.; $P < 0.001$ against initial activity). This decrease was not reversed by 1.4 mM ketoleucine (4.6 ± 0.12, 9 obs.). In rat kidney mitochondria incubation for 30 min with 5 mM oxoglutarate + 0.5 mM L-malate decreased BCDH complex activity from 4.2 ± 0.07 to 2.9 ± 0.17 (mean ± SEM for 4 obs. in units/g mitochondrial protein). This decrease in activity was prevented by 10 μM CCCP (4.1 ± 0.17; 4 obs.) or 1.4 mM ketoleucine (4.3 ± 0.15; 4 obs.).

3.4. *General discussion*

Evidence has been obtained in this study that rat liver and kidney BCDH complexes in extracts of mitochondria are inactivated by phosphorylation with ATP. Inactivation of the liver complex was much slower than that of rat heart complex and only occurred after a lag period. This lag period was removed by preincubation of extracts. The data suggest either an inhibitor of the (presumed) kinase, or need for activation of the kinase, or need for inactivation of the (presumed) phosphatase. The rate of inactivation of the kidney complex was intermediate between that of heart and liver complexes and no lag period was detected. The study has confirmed (cf. [6]) that freshly prepared rat liver and kidney mitochondria contain little, if any, inactive BCDH complex: ⩽20% of BCDH complex in liver and ⩽30% in kidney mitochondria was inactivated during 30 min incubation with respiratory substrates. The results of this study lend further support to the suggestion [6] of tissue specific differences in the interconversion of inactive and active BCDH complexes. It would appear that the inactivating system is present in liver and kidney mitochondria but that its activity may not be expressed. The reason for this has yet to be elucidated. An encouraging feature of these findings is that liver BCDH complex may be used for exploring details of the interconversion of active and inactive BCDH complexes. Rat liver is a more convenient source of BCDH complex than rat muscles or kidney.

Acknowledgements

This investigation was supported by the Medical Research Council and the British Diabetic Association.

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