INACTIVATION OF RAT HEART BRANCHED-CHAIN 2-OXOACID DEHYDROGENASE COMPLEX BY ADENOSINE TRIPHOSPHATE

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1. Introduction

Extracts of freshly prepared rat-liver or ox-liver mitochondria contain active branched-chain 2-oxoacid dehydrogenase complex. This has been partially purified and the specificity, substrate-kinetics and end-product inhibition has been described [1–3]. Although branched chain amino acids are rapidly decarboxylated in perfused rat heart [4] extracts of freshly isolated rat heart mitochondria were found to be very low in branched-chain 2-oxoacid dehydrogenase activity. Evidence is given here that the rat heart complex is inactivated by ATP and that active complex is formed in mitochondria depleted of ATP. It has seemed useful to report these findings because of current interest in the biochemistry and chemical pathology of the complex in animal tissues. Our findings indicate that purification of active complex may require an activation step.

2. Materials and methods

Sources of chemicals and biochemicals were as in [2,5,6].

Rat heart mitochondria were prepared with Nagarse [7]; this method gives minimal contamination with oligomycin-insensitive ATPase. Mitochondria were incubated in KCl medium (2–4 mg protein/ml) at 30°C as in [6]. Other additions are given in the text, table 1 or fig.1. Incubation was terminated by centrifugation for 20 s in an Eppendorf 3200 centrifuge, the supernatant aspirated and the pellets frozen and stored in liquid N_2. Extracts were prepared by thawing the pellets (2 mg protein) into 100 μl extraction medium and mixing in a 250 μl syringe, followed by freezing (liquid N_2) and thawing (20°C). The extraction medium (pH 7.5) was 30 mM potassium phosphate/3 mM EDTA/3 mM 2-mercaptoethanol/5% (v/v) Triton X-100.

Branched-chain 2-oxoacid dehydrogenase complex was assayed by following NAD^+ reduction at 340 nm in a Gilford recording spectrophotometer at 30°C. The assay buffer (0.55 ml, pH 7.5) was 30 mM potassium phosphate/3 mM 2-mercaptoethanol/2 mM $MgCl_2$/0.4 mM CoA/0.4 mM thiamin pyrophosphate/1 mM NAD^+/7.5 mM KCN/0.2 mM 4-methyl 2-oxopentanoate. The assay was initiated with mitochondrial extract (50 μl). A blank (lacking substrate) was run simultaneously with each extract. The inclusion of KCN in the assay medium completely inhibited NADH oxidase activity in mitochondrial extracts. Reaction rate was proportional to volume of extract and progress curves were linear for up to 1 min. The 4-methyl 2-oxopentanoate in the assay medium was replaced by 3-methyl 2-oxopentanoate or 3-methyl 2-oxobutyrate in assays of extracts containing these 2-oxoacids.

Effects of ATP addition on branched-chain 2-oxoacid dehydrogenase activity were investigated in extracts prepared from mitochondria incubated at 30°C for 10 min without substrate (to activate the complex). The procedure was as above except that the extraction buffer (pH 7.5) was 30 mM potassium phosphate/10 mM EGTA/1 mM Tos–Lys–CH_2Cl/5 mM dithiothreitol/25 μg/ml oligomycin/5% (v/v) Triton X-100 (20 mg protein/ml). The complex was stable in such extracts at 0°C for at least 60 min

but activity was lost in 5–10 min incubation at 30°C. Incubations with ATP were therefore made at 0°C and were controlled by parallel incubations without ATP. Control and test samples were then assayed simultaneously for branched-chain 2-oxoacid dehydrogenase complex. Details of additions to the incubation medium are given in the text, table 1 or fig.1.

Pyruvate dehydrogenase complex in mitochondrial extracts was assayed by the same method but with pyruvate in place of 4-methyl 2-oxopentanoate. This method gave values which corresponded closely to those obtained by coupling to arylamine acetyltransferase [8]. One unit of enzyme complex forms 1 μmol NADH/min at 30°C.

3. Results and discussion

The branched-chain 2-oxoacid dehydrogenase complex activity of freshly prepared rat heart mitochondria was low and ranged from 0.11–0.47 munits/mg mitochondrial protein (mean ± SEM 0.26 ± 0.04 for 27 observations on 7 mitochondrial preparations). The activity remained at this low level or was further reduced in mitochondria incubated at 30°C with 5 mM succinate – see table 1 and fig.1 panel (a). Activity of the complex was increased in mitochondria incubated without substrate or by addition of carbonyl cyanide *m*-chlorophenylhydrazone to mitochondria incubated with succinate – see table 1 and fig.1 panel (a). In no substrate incubations, activation was essentially complete in 10 min; the range of activities in mitochondrial preparations were 0.99–2.28 munits/mg mitochondrial protein (mean ± SEM 1.83 ± 0.12 for 27 observations on 7 mitochondrial preparations). These data may show that only 5–15% of branched-chain 2-oxoacid dehydrogenase is active in freshly prepared rat heart mitochondria or after incubation with succinate. Qualitatively similar results were obtained with 3-methyl 2-oxopentanoate and with 3-methyl 2-oxobutyrate as assay substrates (not shown). The complex in rat heart mitochondria is also reactivated in the presence of succinate by incubation with 500 μM 4-methyl 2-oxopentanoate (see table 1) or 50 μM 4-methyl 2-oxopentanoate (quantitatively similar,

Table 1
Activities of branched-chain 2-oxoacid dehydrogenase and pyruvate dehydrogenase complexes in rat heart mitochondria

Mitochondrial incubation	Activity munits/mg protein. Mean ± SEM for	
	Branched-chain 2-oxoacid dehydrogenase complex	Pyruvate dehydrogenase complex
Zero time	0.11 ± 0.06 (4)	79.5 ± 6.5 (3)
No substrate	2.28 ± 0.08[a] (4)	124 ± 3.0[a] (3)
5 mM succinate	0.11 ± 0.04 (4)	28.5 ± 1.5 (3)
5 mM succinate + 50 μM 4-methyl 2-oxopentanoate	2.34 ± 0.03[a] (4)	29.5 ± 1.5 (3)
Zero time	0.29 ± 0.06 (6)	–
No substrate	1.70 ± 0.24[a] (3)	–
5 mM succinate	0.06 ± 0.08 (3)	–
5 mM succinate + 1 μM carbonyl cyanide *m*-chlorophenylhydrazone	0.65 ± 0.08[b] (3)	–

[a] $P<0.01$ against succinate and zero time incubations
[b] $P<0.01$ against succinate incubations

Rat heart mitochondria, prepared with Nagarse, were incubated at 30°C in KCl medium (2.4 mg protein/ml) with additions as shown in column 1. The incubation time was 10 min (except for zero time). Mitochondria were separated by centrifugation, the pellets frozen in liquid N_2 and extracted and extracts assayed for branched-chain 2-oxoacid dehydrogenase and pyruvate dehydrogenase complexes. For further details of preparation, incubation and extraction of mitochondria and spectrophotometric assay of enzyme complexes see section 2. Carbonyl cyanide *m*-chlorophenylhydrazone was added after 2 min of incubation with succinate.

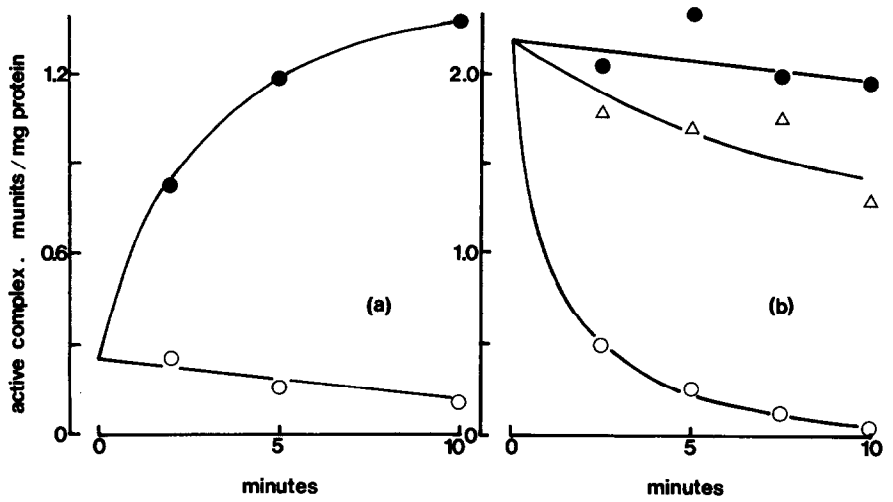

Fig.1. Branched-chain 2-oxoacid dehydrogenase activity in extracts of rat heart mitochondria. Rat heart mitochondria were prepared with Nagarse (see section 2). Panel (a) mitochondria incubated at 30°C in KCl medium for time shown with 5 mM potassium succinate (○) or without substrate (●), separated, extracted and assayed for branched chain 2-oxoacid dehydrogenase complex (see section 2 for details of incubation extraction and assay). Each point is the mean of 18 observations made with 4 mitochondrial preparations; $P<0.001$ for (succinate control) at each period of incubation. Panel (b) extracts prepared from mitochondria incubated at 30°C for 10 min in KCl medium (no substrate). The extracts were incubated at 0°C for the time shown with 0.3 mM ATP (○); or with 0.3 mM ATP + 0.5 mM 4-methyl 2-oxopentanoate (△); or without addition(●). For the symbols (○,●) each point is the mean of at least 5 observations made with at least 4 mitochondrial preparations – $P<0.001$ for (ATP control) at all periods of incubation. (△) Each point is the mean of 2 observations made with 2 mitochondrial preparations.

not shown) or 500 μM 3-methyl 2-oxobutyrate or 500 μM 3-methyl 2-oxopentanoate (quantitatively less effective – not shown).

These results suggested that branched-chain 2-oxoacid dehydrogenase complex is inactivated in rat heart mitochondria by ATP formed by respiratory chain phosphorylation; and that branched-chain 2-oxoacids interfere with inactivation. This conclusion is supported by the results of experiments in which extracts of mitochondria incubated without substrate (to form active complex) were reacted with ATP. These extracts contain Mg at ~0.6 μg-atoms/ml [5] and further Mg was not added. As fig.1 panel (b) shows, incubation with 0.3 mM ATP led over 10 min to a reduction in branched-chain 2-oxoacid dehydrogenase complex activity to ~3% of the control (incubated without ATP). Inactivation was also seen with 0.3 mM ADP but not with 0.3 mM GTP (not shown). The effect of ADP is assumed to be due to ATP formed by adenylate kinase because it could be abolished by inclusion of glucose (10 mM) and yeast hexokinase (4 units/ml). The effect of ADP was also absent with preparations of complex freed of adenylate kinase by centrifugation through a layer of 5 ml 10% (w/v) sucrose for 150 min at 180 000 × *g* in the MSE 75 centrifuge (10 × 10 ml rotor). Such preparations were inactivated by 0.3 mM ATP + 1.0 mM $MgCl_2$ (not shown). When inactive complex formed by incubation of mitochondrial extracts with 0.3 mM ATP was separated from ATP by centrifuging through a 10% sucrose layer, active complex was not reformed (not shown). This may indicate that ATP is not an inhibitor of the active complex. ATP (2 mM) was not an inhibitor in the assay for branched-chain 2-oxoacid dehydrogenase complex (not shown). Panel (b), fig.1 also shows that inactivation of the complex during incubation with 0.3 mM ATP is inhibited by 0.5 mM 4-methyl 2-oxopentanoate. Under the conditions described in fig.1 panel (b), inactivation of the complex by ATP (0.3 mM) was also inhibited by 0.5 mM 3-methyl 2-oxobutyrate or 0.5 mM 3-methyl 2-oxopentanoate (results not shown).

The pyruvate dehydrogenase complex in rat heart mitochondria is inactivated by phosphorylation with

ATP and is activated when mitochondrial ATP is depleted [6]. Evidence that rat liver and ox liver branched-chain 2-oxoacid dehydrogenase complexes are distinct from the pyruvate dehydrogenase complex has been given in [2,3]. There are a number of points of evidence which may show that inactivation of the branched-chain 2-oxoacid dehydrogenase complex by ATP is unrelated to inactivation of the pyruvate dehydrogenase complex by phosphorylation. Pyruvate dehydrogenase complex in the 5% (v/v) Triton extracts of rat heart mitochondria was not inactivated during incubation with ATP. This is an effect of Triton (cf. present data with that in [5]). Data given in the table show that incubation of rat heart mitochondria with succinate + 4-methyl 2-oxopentanoate led to inactivation of the pyruvate dehydrogenase complex whereas the branched-chain 2-oxoacid dehydrogenase complex was activated. It is known that 4-methyl 2-oxopentanoate is only a weak inhibitor of the pig heart pyruvate dehydrogenase kinase reaction [9]. Data in table 1 show also that only 5% of the branched-chain 2-oxoacid dehydrogenase complex was active in freshly prepared mitochondria whereas 64% of the pyruvate dehydrogenase complex was active.

The purpose of this communication is to draw attention to the fact that only a minor proportion of branched-chain 2-oxoacid dehydrogenase complex is active in mitochondria freshly prepared from rat heart. This is in contrast to high activity of the complex in freshly prepared rat liver mitochondria (see section 1). The data presented here do not establish that inactivation of the complex by ATP is due to phosphorylation of the complex. This can only be established by demonstrating incorporation of phosphate into the complex. This is very difficult because of the very low activity of the branched-chain complex relative to the high concentration of pyruvate dehydrogenase complex. Reactivation of the branched-chain complex in extracts of mitochondria has not been detected under conditions which lead to formation of active pyruvate dehydrogenase complex (not shown).

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