# ACTIVE AND INACTIVE FORMS OF BRANCHED-CHAIN 2-OXOACID DEHYDROGENASE COMPLEX IN RAT HEART AND SKELETAL MUSCLE

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## 1. Introduction

Branched-chain 2-oxoacid dehydrogenase complex activity is low in freshly prepared rat heart mitochondria as compared with rat liver or bovine kidney or liver mitochondria [1–4]. The low activity of the heart complex is apparently due to reversible inactivation by MgATP [1]. Thus activity of the complex is increased up to 20-times when heart mitochondria are depleted of ATP by incubation without respiratory substrate or with uncouplers of respiratory chain phosphorylation. Low activities persist in mitochondria incubated with respiratory substrates. Active complex in extracts of mitochondria is inactivated by incubation with MgATP; this inactivation is prevented by branched-chain 2-oxoacids and branched-chain 2-oxoacids reactivate the complex in rat heart mitochondria [1]. Purified bovine kidney complex is not inactivated by MgATP [4]. These observations suggested that there are interconvertible active and inactive forms of the complex in rat heart but not in rat liver or kidney or bovine kidney. Evidence is given here for inactivation of branched-chain complex in rat heart and skeletal muscle mitochondria by ATP; for reactivation of inactive complex in extracts of heart mitochondria by preparations of pyruvate dehydrogenase phosphate phosphatase; for interconvertible active and inactive forms of the complex in perfused rat heart; and for absence of complex inactivation by ATP in rat liver or kidney mitochondria.

*Abbreviations:* TPP, thiamin pyrophosphate; OIC, 2-oxoisocaproate (4-methyl, 2-oxopentanoate); TLCK, *N*-α-*p*-tosyl-1-lysine chloromethyl ketone; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DTT, dithiothreitol; EGTA, ethanedioxybis-(ethylamine)-tetra acetate

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## 2. Experimental

Sources of biochemicals and chemicals are given in [1–3]. Pyruvate dehydrogenase phosphate phosphatase was partially purified from ox-hearts [5]. Rat heart mitochondria were prepared with Nagarse [6]; rat psoas muscle mitochondria were prepared as in [7]. Rat kidney mitochondria were prepared as in [8]; rat liver mitochondria were prepared by the same method except that the first mitochondrial pellet was incubated for 2 min at 4°C with digitonin (1 mg/ml; 5 ml/liver) to remove lysosomes [9].

Rat hearts were perfused by drip through [10] at 38°C with bicarbonate-buffered saline saturated with 95% $O_2$/5% $CO_2$; for other additions and time of perfusion see section 3.4. Hearts were frozen with a tissue clamp at liquid $N_2$ temperature and powdered under liquid $N_2$ with a pestle and mortar. After warming to −10°C powder from each heart was extracted into 5 ml 30 mM potassium phosphate/3 mM EDTA/5 mM DTT/0.5 mM OIC/1 mM TLCK/5% (v/v) Triton X-100/pH 7.5 by vortex-mixing and freezing (liquid $N_2$) and thawing (30°C) 3 times. The extracts were centrifuged (2°C) at 33 000 × *g* for 5 min to remove debris; the supernatants were then centrifuged at 180 000 × *g* for 90 min to concentrate the branched-chain complex. The pellets containing the pyruvate, 2-oxoglutarate and branched-chain 2-oxoacid dehydrogenase complexes were each taken up in 0.5 ml 30 mM potassium phosphate/5 mM DTT/pH 7.5 with an all glass Potter-Elvehjem homogeniser.

Mitochondria were incubated in Eppendorf cen-

trifuge tubes at 30°C in KCl medium [10] (1–4 mg protein/ml), separated by centrifugation and frozen as in [10]. Extracts were prepared by freezing and thawing in extraction medium containing (unless stated) 5% Triton X-100 as in [1].

Branched-chain 2-oxoacid dehydrogenase complex activity was assayed in extracts of rat heart or mitochondria as the initial rate of $NAD^+$ reduction at 30°C, measured spectrophotometrically at 340 nm. Assay buffer and conditions of assay were as in [1]. A blank correction was subtracted (rate of $\Delta E_{340}$ in the absence of OIC measured concurrently). Pyruvate and 2-oxoglutarate dehydrogenase complex activities were assayed by the same method but with 1 mM pyruvate or 1 mM 2-oxoglutarate. Rates were proportional to volume of extract and constant for ~1 min. Citrate synthase activity was assayed as in [10].

A unit of enzyme activity forms 1 μmol product/min at 30°C. Values of $K_m$ were computed as in [11] and concentrations of free $Mg^{2+}$ and $Ca^{2+}$ as in [5]. The proportion of branched-chain complex in the active form was calculated as follows. Assays in extracts of perfused heart gave the activity ratio of (active branched-chain complex)/(2-oxoglutarate dehydrogenase complex) ($A$). Assays in extracts of mitochondria incubated for 15 min at 30°C (to effect conversion of inactive complex into active complex) gave the activity ratio (total branched-chain complex)/(2-oxoglutarate dehydrogenase complex) ($B$). The % of active complex was calculated as $100\,A/B$. This method was chosen because conversion of inactive complex to active complex in heart extracts has not been satisfactorily achieved. The 2-oxoglutarate dehydrogenase complex was used as marker enzyme because it co-sedimented with the branched-chain complex in a linear 5–20% (w/v) sucrose gradient (not shown) and because it does not exist in active and inactive forms. The method assumes equivalent recoveries of the two complexes.

## 3. Results and discussion

### 3.1. $K_m$ values for rat heart branched-chain 2-oxoacid dehydrogenase complex

These experiments were made with extracts of mitochondria incubated in bulk at 30°C for 15 min in KCl medium, separated by centrifugation (5 min, 33 000 × $g$), frozen, and extracted as in section 2. The $K_m$ values were (mean ± SEM) 14.5 ± 6.0 μM OIC (8 obs.; 6 conc. from 5–200 μM); 10.6 ± 2.3 μM CoA (8 obs.; 6 conc. from 12.5–400 μM); 40.0 ± 5.7 μM $NAD^+$ (8 obs.; 6 conc. from 31.25 μM–1 mM). These values are close to those for rat liver complex [3].

### 3.2. Effect of intramitochondrial ATP concentration on activity of branched-chain 2-oxoacid dehydrogenase complex in rat heart, psoas muscle, liver and kidney mitochondria

Freshly prepared rat heart mitochondria have low branched-chain complex activity which persists on incubation with respiratory substrate (e.g., 5 mM succinate) [1]. Activity is increased up to 20 times by depletion of mitochondrial ATP (uncouplers or incubation at 30°C without substrate) or incubation with OIC [1] or 3-methyl-2-oxopentanoate or 3-methyl-2-oxobutyrate (P.J.P., P.J.R., unpublished). As shown in table 1 (lines 1–3) the activity of the branched-chain complex in rat heart mitochondria was reduced to 2% by extramitochondrial ATP under conditions where ATP may enter mitochondria in exchange for ADP (presence of oligomycin and CCCP). This effect of external ATP was blocked by 0.5 mM OIC. In these experiments the complex was activated by 10 min of pre-incubation in KCl medium with oligomycin + CCCP before additions of ATP ± OIC. In rat psoas muscle mitochondria incubated with 5 mM succinate, branched-chain complex activity was increased 7-fold by 10 μM 2,4-dinitrophenol (table 1, lines 4–7). This observation supports the suggestion that the rat skeletal muscle complex [12] like rat heart complex is inactivated by ATP.

The activity of branched-chain complex in rat liver mitochondria incubated with oligomycin and CCCP was not decreased by ATP; the activity of the pyruvate dehydrogenase complex in the same experiment was decreased to 22% of the control by ATP (table 1, lines 8–9). In rat liver mitochondria (lines 10,11) or rat kidney mitochondria (lines 12,13) incubated with 5 mM succinate, 10 μM 2,4-dinitrophenol did not increase branched-chain complex activity. Pyruvate dehydrogenase complex activity measured under the same conditions increased with dinitrophenol 3.5 times in liver mitochondria and 3.2 times in kidney mitochondria. These results indicate that rat liver and rat kidney branched-chain 2-oxoacid dehydrogenase complexes are not inactivated by ATP in mitochondria in contradistinction to results with rat heart and rat psoas muscle mitochondria.

Table 1
Effect of external ATP, or of succinate or 2,4-dinitrophenol on activities of branched-chain 2-oxoacid and pyruvate dehydrogenase complexes in rat heart, psoas, liver and kidney mitochondria

| Mitochondria | Added to KCl medium | Activity; mean ± SEM in munits complex/unit citrate synthase | |
|---|---|---|---|
| | | Branched-chain 2-oxoacid dehydrogenase complex | Pyruvate dehydrogenase complex |
| Rat heart | None | 1.67 ± 0.06* (3)[b] | – |
| | 5 mM-ATP | 0.03 ± 0.03 (3)[b] | – |
| | 5 mM-ATP + 0.5 mM-OIC | 1.39 ± 0.03* (3)[b] | – |
| Rat psoas | (as made) | 0.16 ± 0.04* (20) | – |
| | 5 mM-succinate | 0.15 ± 0.10* (8)[a] | – |
| | | 0.21 ± 0.05* (11)[b] | – |
| | 10 μM-2,4-dinitrophenol | 1.13 ± 0.06 (19)[b] | – |
| Rat liver | None | 31.0 ± 0.7 (3)[a] | 129.0 ± 3.6 (3)[a] |
| | 10 mM-ATP | 30.5 ± 1.4 (3)[a] | 29.7 ± 2.4* (3)[a] |
| | 5 mM-succinate | 15.0 ± 2.2 (3)[a] | 14.7 ± 4.2 (3)[a] |
| | 5 mM-succinate + 10 μM-2,4-dinitrophenol | 15.8 ± 0.8 (3)[a] | 101.0 ± 2.4* (3)[a] |
| Rat kidney | 5 mM-succinate | 7.9 ± 0.80 (3)[b] | 40.2 ± 4.2 (3)[b] |
| | 5 mM-succinate + 10 μM-2,4-dinitrophenol | 7.1 ± 0.59 (3)[b] | 129.4 ± 2.0* (3)[b] |

Rat heart *$P < 0.01$ against 5 mM ATP; psoas *$P < 0.01$ against 2,4-dinitrophenol; liver *$P < 0.01$ for effect of ATP, and of 2,4-dinitrophenol; kidney *$P < 0.01$ for effect of 2,4-dinitrophenol

Mitochondria were incubated in KCl medium (see section 2) at 30°C for 5 min[a] or 10 min[b] with additions as shown, separated by centrifugation, frozen and extracted. Extracts were assayed for branched-chain 2-oxoacid and pyruvate dehydrogenase activities and citrate synthase activity. In experiments in which effects of ATP addition were studied, KCl medium contained EDTA in place of EGTA and additionally 10 μM CCCP and oligomycin (25 μg/ml). In experiments with rat heart mitochondria only, mitochondria were preincubated in KCl medium for 10 min at 30°C before making additions as shown and commencing incubation. For details of preparation of mitochondria, composition of KCl medium and extraction and assays see section 2. No. obs. in parentheses

### 3.3. *Reactivation of inactive rat heart branched-chain 2-oxoacid dehydrogenase complex by partially purified ox-heart pyruvate dehydrogenase phosphate phosphatase*

Reactivation of inactive rat heart mitochondrial branched-chain complex by a preparation of ox-heart pyruvate dehydrogenase phosphate phosphatase is shown in fig.1. In (a) inactive complex was obtained by extracting freshly prepared rat heart mitochondria. In these mitochondria ~30% of complex was inactive (based on reactivation by 12 min incubation at 30°C in KCl medium). Complete reactivation of the complex in extracts was achieved by 20–25 min incubation with phosphatase in the presence of $MgCl_2$ (free $Mg^{2+}$, 1.3 mM) and CaEGTA buffer (free $Ca^{2+}$, 13.6 μM). No reactivation was seen in the absence of phosphatase, or in the absence of $Mg^{2+}$ and at very low $Ca^{2+}$ (absence of $MgCl_2$; 10 mM EDTA; computed $Ca^{2+}$, 0.08 μM). Active complex, in extracts of preincubated mitochondria showed a small activation with phosphatase and no change of activity in the absence of phosphatase.

In fig.1 (b) inactive complex was obtained by incubating extracts of rat heart mitochondria (in

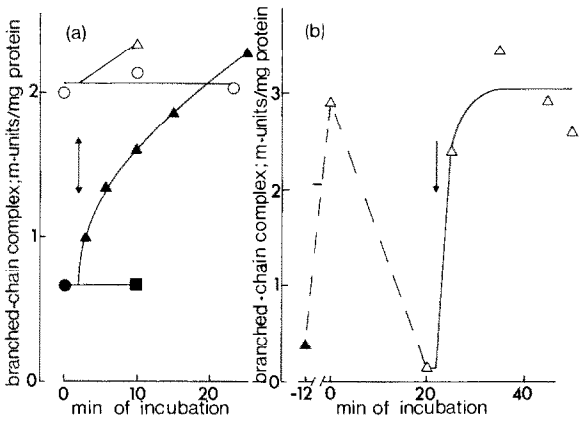


Fig.1. Branched-chain 2-oxoacid dehydrogenase complex activity in extracts of rat heart mitochondria.
(a) Closed symbols extracts of freshly prepared mitochondria. Open symbols, extracts of mitochondria incubated for 12 min at 30°C in KCl medium without respiratory substrate. Extracts prepared in medium without Triton X-100 (see section 3.3). Extracts (13.4 mg protein/ml) were incubated at 30°C with the following additions after 2 min (arrows); (○,●) none; (△,▲) $MgSO_4$ to 10 mM + 0.1 vol. ox-heart pyruvate dehydrogenase phosphatase; (■) $MgSO_4$ to 10 mM, (■) EDTA to 10 mM + 0.1 vol phosphatase. Samples were taken for assay of branched-chain 2-oxoacid dehydrogenase complex at times shown; in these experiments assay buffer (see section 2) contained 5% (v/v) Triton X-100. Each point is mean of 2 obs.
(b) Freshly prepared rat heart mitochondria (▲) were preincubated for 12 min at 30°C in KCl medium without respiratory substrate. Extracts (21.2 mg protein/ml) were then prepared in medium without Triton X-100 (see section 2) and (△) incubated for 20 min at 0°C with 0.3 mM ATP; then 0.1 vol. (100 mM EGTA, 99 mM $CaCl_2$, 100 mM $MgSO_4$, 2 mM TPP, 5 mM OIC) added and the incubation mixture incubated at 30°C for 2 min. Pyruvate dehydrogenase phosphatase (0.1 vol.) was then added (arrow). Each point is mean of 2 obs.

which complex had been activated by preincubation) with 0.3 mM ATP for 20 min at 0°C (the $Mg^{2+}$ required for this reaction was derived from the mitochondria). After warming to 30°C addition of phosphatase + $MgCl_2$ + CaEGTA buffer ($Mg^{2+}$ and $Ca^{2+}$ concentrations as in fig.1 (a)) plus OIC to 0.5 mM (to inhibit the inactivation reaction [1]) led to complete reactivation within 15 min. No reactivation was seen in the absence of phosphatase (not shown).

In these experiments mitochondrial extracts were made in 30 mM potassium phosphate/5 mM DTT/1 mM TLCK/0.2 mM TPP/10 mM EGTA/9.9 mM $CaCl_2$/pH 7.5 (fig.1 (a)); or 30 mM potassium phosphate/5 mM DTT/1 mM TLCK/pH 7.5 (fig.1 (b)); with other additions as in (a) after 20 min incubation with ATP). The active branched-chain complex is stable on incubation at 30°C under these conditions because of the absence of Triton (cf. [1]). Stability was improved with TPP (not shown).

These results show that an ox-heart pyruvate dehydrogenase phosphate phosphatase preparation converted inactive branched-chain 2-oxoacid dehydrogenase complex into the active form under conditions (i.e., presence of $Mg^{2+}$ and $Ca^{2+}$) which leads to conversion of pyruvate dehydrogenase phosphate complex into its active (dephosphorylated) form [13].

### 3.4. *Proportion of active branched-chain 2-oxoacid dehydrogenase complex in perfused rat heart*

These data are shown in fig.2. In hearts of fed normal rats ~13% of branched-chain complex was in the

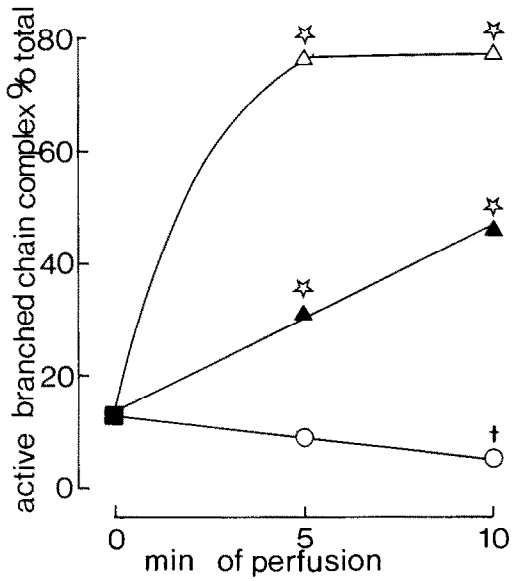


Fig.2. Branched-chain 2-oxoacid dehydrogenase complex activity in perfused rat heart. Hearts of fed normal rats were perfused by drip through at 38°C with bicarbonate-buffered saline gassed with ($O_2$/$CO_2$: 95/5) and containing; (○) 5.5 mM glucose; (▲) 5.5 mM glucose + 50 μM OIC; (△) 5.5 mM glucose + 0.5 mM OIC. At the times shown the heart was clamped at liquid $N_2$ temperature, powdered under liquid $N_2$ and extracted and processed as in section 2. The activities of branched-chain 2-oxoacid (active form) and 2-oxoglutarate dehydrogenase complexes were assayed and the % of active complex calculated as in section 2. Each point is mean of 3 obs. The total activity of the branched-chain complex was 0.16 units/g wet wt of heart.
†$P < 0.05$ against zero time. *$P < 0.01$ against (○).

active form; this decreased to ~6% after 10 min perfusion with 5 mM glucose. The proportion of active complex was increased when OIC was present in addition to glucose to reach 50% (50 μM OIC; 10 min perfusion) or 77% (500 μM OIC; 5 or 10 min perfusion). With 0.5 mM L-leucine + 5.5 mM glucose the proportion of active complex was 22.6% (at 6 min; not shown; $P<0.02$ against glucose alone). The proportion of active complex was thus increased by OIC and by leucine, its precursor in vivo. These results would appear to show that inactivation of the complex by ATP occurs in vivo; that this inactivation is inhibited by OIC in vivo; and that reactivation occurs in vivo.

The highest total activity of branched chain complex in rat heart was 0.30 unit/g wet wt measured at 30°C (assuming 80 mg mitochondrial protein/g wet wt). Rat heart perfused with L-leucine at 38°C shows oxidation rates of the amino acid which are approximately proportional to perfusate concentration up to a maximum of 0.12 μmol . $min^{-1}$ . g wet $wt^{-1}$ at 1 mM [14]. It may thus be inferred that the rate of oxidation was 0.06 μmol . $min^{-1}$ . $g^{-1}$ at 0.5 mM. Our estimates of branched-chain complex activity (0.07 unit at 30°C with 0.5 mM L-leucine) may thus be consistent with measured rates of leucine oxidation in the perfused heart at 38°C.

### 3.5. *General discussion*

In first describing interconvertible active and inactive forms of the branched-chain complex in rat heart mitochondria and inactivation by ATP [1] we were unable to show reactivation in mitochondrial extracts. This has been accomplished here by adding a preparation of ox-heart pyruvate dehydrogenase phosphate phosphatase and by defining conditions where branched-chain complex in extracts of mitochondria is stable at 30°C. This evidence, in conjunction with the observation that non-phosphorylating analogues of ATP do not inactivate the complex in extracts of skeletal muscle [12] or rat heart mitochondria (P.J.P., P.J.R., unpublished), suggests strongly that inactivation is due to phosphorylation. We have shown further that active and inactive forms of the complex are present in heart in vivo; and that OIC (and its precursor leucine) effect conversion of inactive complex into active complex. The interconversion may therefore be of physiological significance.

This study and [1,4,12] indicate that interconvertible active and inactive forms of the complex are present in heart and skeletal muscles but not in liver or kidney mitochondria. This provides for the possibility of differential regulation of branched-chain amino acid oxidation in muscle as compared with liver and kidney. This could, for example, be related to their unique capacity to form glucose (liver and kidney) or ketone bodies (liver) from individual branched-chain amino acids. There may be mechanisms whereby branched-chain amino acids may either be directly oxidised in the periphery or indirectly through formation of ketone bodies and glucose in liver (and kidney). These questions may be answered by regional measurements of clearance rates of branched-chain 2-oxoacids in vivo.

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