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ACETOACETATE ACTIVATION AND OXIDATION IN KIDNEY AND HEART MITOCHONDRIA

A. ALEXANDRE, D. SILIPRANDI AND N. SILIPRANDI
Institute of Biological Chemistry, University of Padova, Padova (Italy)
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SUMMARY

The conditions under which acetoacetate is oxidized by kidney and heart mitochondria have been studied. By means of specific inhibitors it has been possible to study the organization of acetoacetate-activating systems *i.e.*, the 3-ketoacid CoA-transferase reaction and the CoA ligase reaction. The former reaction is arsenate sensitive and ATP independent, the latter, which is ATP dependent, is compartmented in two different sites. The oxidation of acetoacetyl-CoA produced at one of these sites is carnitine dependent and appears to be disconnected from the oxidation site.

Preliminary evidence has been obtained that the enzyme responsible for the reversible transfer of the acetoacetyl radical from carnitine to CoA is identifiable with the short-chain acetylcarnitine transferase.

INTRODUCTION

Acetoacetate is metabolized by several mammalian tissues in different ways. Although liver mitochondria efficiently synthesize acetoacetate, there is virtually no capability for the oxidation of acetoacetate[1]. In kidney and heart mitochondria, on the other hand, the presence of 3-ketoacid CoA-transferase (succinyl-CoA:3-oxoacid CoA-transferase, EC 2.8.3.5)[2] permits rapid oxidation of acetoacetate.

In the present communication evidence is presented indicating that direct acetoacetate activation is possible in mitochondria from kidney and heart.

MATERIALS AND METHODS

Mitochondria from 250–300 g Wistar strain albino rats were prepared essentially by the procedure of SCHNEIDER[3]. Heart and kidney mitochondria were isolated in 0.25 M sucrose containing $2 \cdot 10^{-2}$ M EDTA (pH 7.4). The chelating agent was omitted in the final suspension which consisted only of 0.25 M sucrose. Rats were fasted 15 h before the livers were removed.

Oxygen uptake was measured with a Clark oxygen electrode as described by KIELLEY AND BRONK[4], or manometrically using differential manometers.

Carnitine acetyltransferase (EC 2.3.1.7) was prepared from pigeon breast muscle by the procedure of CHASE *et al.*[5].

The rate of generation of acetyl-CoA from acetylcarnitine and CoA was measured using the citrate synthase assay system of OCHOA[6]. Cuvettes contained, in a final volume of 1 ml: 100 μmoles of Tris–HCl buffer (pH 8.0), 4 μmoles of EDTA, 1 μmole of NAD^+, 4.5 μmoles of DL-malate, 0.2 μmole of CoA, 5 units of malate dehydrogenase (EC 1.1.1.37) and 0.14 unit of citrate synthase (EC 4.1.3.7). The reaction was started by the addition of the transferase preparation and of acetyl-(−)-carnitine. The increase in absorbance at 340 mμ was followed.

The rate of generation of acetoacetyl-CoA from acetoacetyl-(−)-carnitine and CoA was measured by following NADH disappearance in a coupled system with 3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35). Cuvettes contained, in a final volume of 1 ml: 100 μmoles of Tris–HCl buffer (pH 8.0), 4 μmoles of EDTA, 0.2 μmole of NADH, 0.2 μmole of CoA and 0.6 unit of 3-hydroxyacyl-CoA dehydrogenase. The reaction was started by the addition of an active transferase preparation and of acetoacetyl-(−)-carnitine. The decrease in absorbance at 340 mμ was followed.

Mitochondrial ATP was measured by the method of LAMPRECHT AND TRAUTSCHOLD[7].

Acetoacetate was determined by the method of WALKER[8].

Acetoacetyl-(−)-carnitine and acetoacetyl-(+)-carnitine, as well as acetyl-(−)-carnitine and acetyl-(+)-carnitine were gifts of Italseber SPA, Milano. They proved to be chromatographically pure in silica gel thin-layer chromatography run at room temperature with methanol–chloroform–ammonia (40:35:25, by vol.).

Malate dehydrogenase, citrate synthase, 3-hydroxyacyl-CoA dehydrogenase, hexokinase and glucose-6-phosphate dehydrogenase were from Boehringer, Mannheim.

Atractyloside was kindly given by Professor Franco Piozzi of the University of Palermo, Italy.

RESULTS

Fig. 1 (Trace A) shows that kidney mitochondria in the presence of oxaloacetate and 2,4-dinitrophenol readily oxidized acetoacetate. Arsenate, in the absence of added inorganic phosphate, induced an immediate inhibition. It is conceivable that under these conditions acetoacetate activation is sustained by an ATP-independent activation process *via* the 3-ketoacid CoA-transferase reaction. It is likely that arsenate, which is known to induce a rapid hydrolysis (arsenolysis) of succinyl-CoA (ref. 9), might in this way prevent the reversible transfer of CoA from succinyl-CoA to acetoacetate.

In the presence of arsenate, that is with the 3-ketoacid CoA-transferase apparently blocked (Fig. 1, Trace B), acetoacetate-dependent respiration could be restored by the combined addition of ATP and oligomycin. The ATP dependence indicates that a CoA ligase reaction may be operative.

By simultaneously inhibiting the 3-ketoacid CoA-transferase reaction (through the addition of arsenate) and the CoA ligase reaction (by addition of atractyloside which prevents the permeation of ATP through the inner mitochondrial membrane[10–13]) the oxidation of acetoacetate could be restored by the combined addition of CoA, carnitine and inorganic phosphate (Fig. 1, Trace C). Inorganic phosphate alone was ineffective in restoring oxidation. The oxidation of acetoacetate, which is

dependent on added CoA and carnitine, can be explained by assuming an external acetoacetate CoA ligase reaction linked to an acetoacetyl-CoA–carnitine exchange reaction. Similar results have been obtained with heart mitochondria.

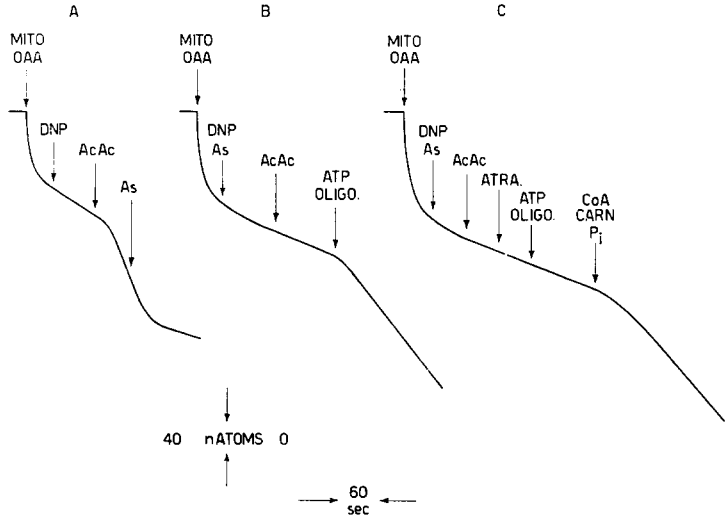

Fig. 1. Acetoacetate oxidation in kidney mitochondria. The incubation mixture contained 10 mM Tris–HCl (pH 7.4), 26 mM NaCl, 58 mM KCl, 6 mM $MgCl_2$. Total volume, 2 ml; temp., 22°. The following were added at the points indicated by arrows: 8 mg of kidney mitochondrial protein (MITO), 0.25 μmole of oxaloacetate (OAA), 0.05 μmole of 2,4-dinitrophenol (DNP), 7 μmoles of arsenate (As), 5 μmoles of acetoacetate (AcAc), 10 μg of oligomycin (OLIGO.), 7 μmoles of ATP, 1.5 μmoles of atractyloside (ATRA.), 0.3 μmole of CoA, 1 μmole of DL-carnitine (CARN.) and 30 μmoles of P_i.

From Table I it can be observed that oxygen uptake, measured in a Warburg apparatus over a longer period of time, in the presence of acetoacetate as substrate, together with sparking amount of oxaloacetate, was maximal under conditions (addition of 2,4-dinitrophenol) in which only the 3-ketoacid CoA-transferase reaction was presumed to occur. Under conditions in which the 3-ketoacid CoA-transferase

TABLE I

STOICHIOMETRY OF ACETOACETATE DISAPPEARANCE AND OXYGEN CONSUMPTION IN KIDNEY MITOCHONDRIA

Incubation mixture as for Fig. 1, with the further addition of 5 mM acetoacetate, 0.12 mM oxaloacetate, 0.025 mM 2,4-dinitrophenol. Where indicated, arsenate (5 μmoles), ATP (2 μmoles) and oligomycin (5 μg) were added. Kidney mitochondrial protein 5 mg. Total vol. 1 ml. Temperature 25°. Incubation time 30 min.

Additions	*Acetoacetate disappearance (μmoles)*	*Oxygen uptake (μatoms)*
None	2.70	17.71
Arsenate	0	0.65
Arsenate + oligomycin + ATP	0.75	5.41

reaction was inhibited (addition of 2,4-dinitrophenol + arsenate + ATP + oligomycin) and only the CoA ligase reaction was presumably running, oxygen uptake was lower but still significant, whereas it was negligible when both the 3-ketoacid CoA-transferase and CoA ligase reactions were inhibited (addition of 2,4-dinitrophenol + arsenate).

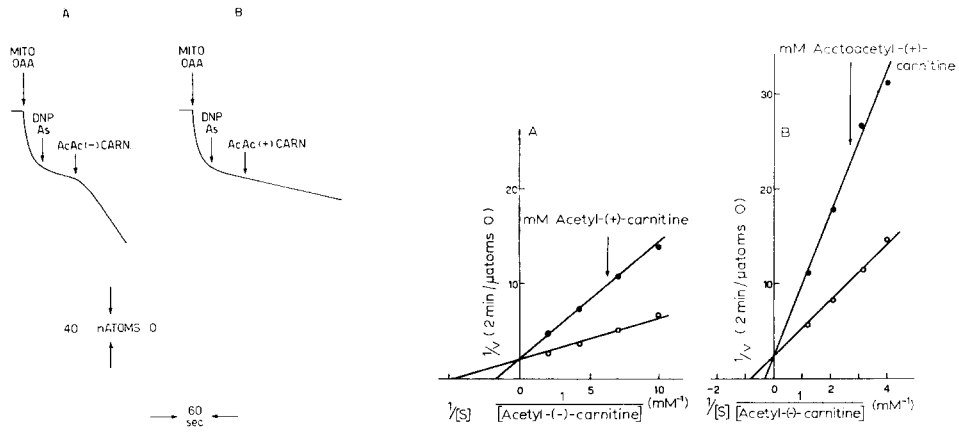

Fig. 2. Acetoacetylcarnitine oxidation in kidney mitochondria. Incubation conditions as for Fig. 1. At the points indicated by arrows the following were added: 8 mg of kidney mitochondrial protein, 0.25 µmole of oxaloacetate, 0.03 µmole of 2,4-dinitrophenol, 7 µmoles of arsenate, 4 µmoles of acetoacetyl-(−)-carnitine (Trace A) and 4 µmoles of acetoacetyl-(+)-carnitine (Trace B). Abbreviations: see legend to Fig. 1.

Fig. 3. Effect of acetyl-(+)-carnitine on acetyl-(−)-carnitine oxidation (A), and of acetoacetyl-(+)-carnitine on acetoacetyl-(−)-carnitine oxidation (B), in rat heart mitochondria. Heart mitochondria (3.8 mg protein) were incubated at 25° in a medium containing: 9.75 mM K_2HPO_4, 2.25 mM KH_2PO_4, 9 mM NaF, 19.5 mM NaCl, 43.5 mM KCl, 5 mM $MgCl_2$, 1.5 mM ADP, 0.25 mM oxaloacetate. Final volume, 2.0 ml.

From the same Table I it appears that disappearance of acetoacetate always paralleled oxygen uptake.

As shown in Fig. 2 (Trace A), acetoacetyl-(−)-carnitine, unlike acetoacetate, was oxidized by kidney mitochondria even in the presence of arsenate. The (+) isomer was not oxidized (Trace B).

The influence of the unnatural (+) isomer of acetyl carnitine on the oxidation of acetyl-(−)-carnitine by heart mitochondria was examined. In Fig. 3A, the reciprocal of the acetyl-(−)-carnitine concentration in the presence and in the absence of acetyl-(+)-carnitine was plotted *versus* the reciprocal of the oxidation rate. It appears that acetyl-(+)-carnitine behaves as a competitive inhibitor of acetyl-(−)-carnitine oxidation. In Fig. 3B, a similar competitive inhibition of acetoacetyl-(−)-carnitine oxidation by acetoacetyl-(+)-carnitine is shown. CHASE AND TUBBS[14] reported a different inhibitory effect of acetyl-(+)-carnitine on acetyl-(−)-carnitine metabolism. Since their experiments were performed with a highly purified enzyme preparation, the two results are probably not directly comparable.

It was also found that acetyl-(−)-carnitine oxidation in rat heart mitochondria is competitively inhibited by acetoacetyl-(+)-carnitine (Fig. 4A), and *vice versa* (Fig. 4B).

These results are consistent with the hypothesis that the transferase reaction between acetoacetyl-(—)-carnitine and CoA yielding acetoacetyl-CoA *plus* free carnitine is catalyzed by the same enzyme which exchanges CoA with acetylcarnitine. This enzyme has also been shown by CHASE[15] to exhibit a very wide substrate specificity.

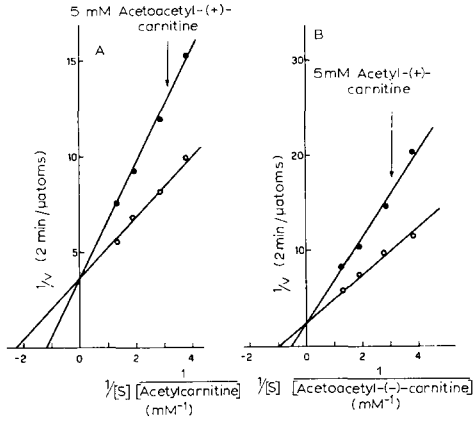

Fig. 4. Effect of acetyl-(+)-carnitine on acetoacetyl-(—)-carnitine oxidation (A), and of acetoacetyl-(+)-carnitine on acetyl-(—)-carnitine oxidation (B), in rat heart mitochondria. Incubation mixture as for Fig. 3.

Preparations of acetylcarnitine transferase from pigeon breast muscle were carried out according to CHASE *et al.*[5]. Fractions were tested during the purification with acetoacetyl-(—)-carnitine or with acetyl-(—)-carnitine as substrates. The relative activities with the two substrates were constant throughout, with the rate of acetyl-(—)-carnitine conversion about 1.4 times that of acetoacetyl-(—)-carnitine. The K_m for acetyl-(—)-carnitine and for acetoacetyl-(—)-carnitine with the purified enzyme was 0.1 and 0.33 mM, respectively.

DISCUSSION

The two fundamental mechanisms for acetoacetate activation, namely, the 3-ketoacid CoA-transferase and CoA ligase reactions, were exhibited in intact rat kidney and heart mitochondria. The 3-ketoacid CoA-transferase reaction, which is ATP independent, proceeds spontaneously in the presence of 2,4-dinitrophenol. This reaction is inhibited by arsenate, which is known to produce arsenolysis of succinyl-CoA (ref. 9) (Fig. 1, Trace A).

In an arsenate-inhibited system it is possible to demonstrate an ATP-linked CoA ligase on addition of ATP and oligomycin. From the results reported in Table I it appears that acetoacetate activation occurs predominantly through the 3-ketoacid CoA-transferase reaction and significantly also through the CoA ligase reaction. Since the CoA ligase reaction is specifically inhibited by atractyloside (Fig. 1, Trace C), it is concluded that the CoA ligase responsible for acetoacetate activation is operative internally to the atractyloside barrier ("internal CoA ligase"). Moreover a second acetoacetate CoA ligase ("external CoA ligase") is located externally to the atrac-

tyloside barrier. Indeed, as depicted in Fig. 5, in the atractyloside-inhibited system the oxidation of acetoacetate is still possible provided that CoA, ATP, inorganic phosphate and carnitine are added (Fig. 1, Trace C).

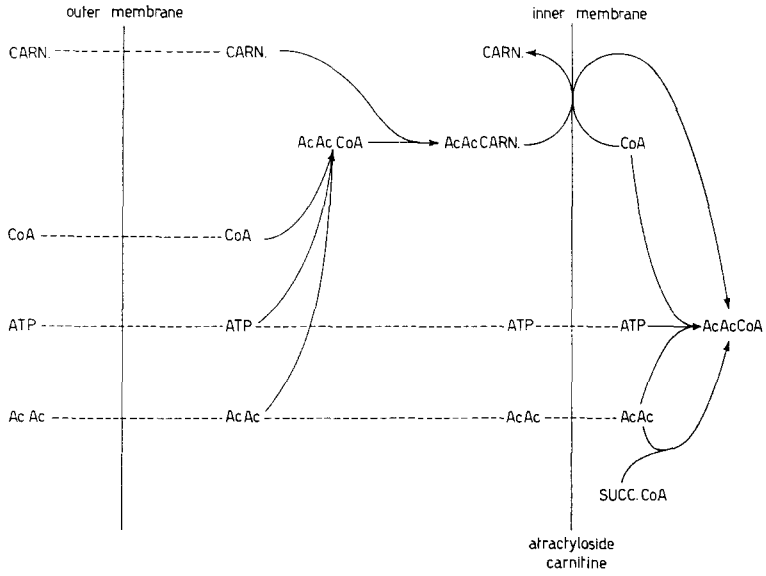

Fig. 5. Proposed scheme for the organization of acetoacetate activation in kidney mitochondria. CARN., carnitine; AcAc, acetoacetate; SUCC.CoA, succinyl-CoA.

Such a situation resembles that already described for long-chain fatty acids in liver[16–20] and kidney mitochondria[21], and implies an exchange reaction between acetoacetyl-CoA, which is formed outside the atractyloside barrier, and carnitine (carnitine acetoacetyltransferase). Acetoacetylcarnitine, unlike acetoacetyl-CoA, permeates through the mitochondrial barriers and transports the activated acetoacetyl radical to the site of its oxidation.

The present research makes it likely that carnitine acetoacetyltransferase, an enzyme which has a prominent position in the metabolic pathway of acetoacetate metabolism, is identical with carnitine acetyltransferase. The identity of the two enzymes appeared both from the cross inhibition experiments in intact heart mitochondria (Fig. 4) and from experiments with the purified enzyme from pigeon breast muscle.

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