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ON THE OXIDATION OF α-OXOBUTYRATE BY ISOLATED MAMMALIAN MITOCHONDRIA

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SUMMARY

The oxidation of α-oxobutyrate in rat kidney and liver mitochondria depleted of endogenous ATP has been studied. The use of malonate and atractyloside as inhibitors made it possible to identify the compartmentation of the successive steps in the mitochondrial structure. It is suggested that oxidative decarboxylation of α-oxobutyrate occurs only inside the atractyloside-sensitive barrier (*i.e.* the inner membrane), while the carboxylation step *i.e.* the carboxylation of propionyl-CoA to methylmalonyl-CoA may occur either inside or outside this barrier.

INTRODUCTION

Previous work on methionine oxidation by kidney and liver mitochondria[1] and the knowledge that α-oxobutyrate is the major intermediate in the oxidative pathway of this amino acid[2], led us to investigate the proper conditions under which α-oxobutyrate is utilized by kidney and liver mitochondria. Since 1955, KINNORY, TAKEDA AND GREENBERG[3], have shown that homogenate obtained from pigeon liver catalyzes the oxidative decarboxylation of α-oxobutyrate to propionate, presumably to propionyl-CoA. The oxidation of propionyl-CoA then proceeds *via* methylmalonyl-CoA[4], succinyl-CoA[5] and finally *via* the Krebs tricarboxylic cycle.

In the present paper are reported some detailed experiments on the optimal conditions for the oxidation of α-oxobutyrate, in order to determine the role of P_i, ATP, carnitine and other cofactors in the over-all process of oxidation of this substrate. In addition, the possible compartmentation of the enzymes involved in the different metabolic steps is discussed.

MATERIALS AND METHODS

Mitochondria were isolated from kidney and liver of Wistar strain albino rats by the conventional 0.25 M sucrose procedure of SCHNEIDER AND HOGEBOOM[6].

O_2 consumption was measured with the Clark electrode essentially as described by KIELLEY AND BRONK[7]; alternatively O_2 uptake was measured manometrically in Barcroft flasks and manometers[8].

α-Oxobutyrate was determined enzymatically by measuring the disappearance of NADH in a system containing 10 mM Tris–HCl (pH 7.3), $1.5 \cdot 10^{-4}$ M NADH and an excess of purified lactate dehydrogenase (EC 1.1.2.3), according to BÜCHER *et al.*[9] and GRUNAU AND GUNSALUS[10]. Protein was determined by a biuret method[11].

All reagents used were analytical grade. Sodium α-oxobutyrate was purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.), sodium propionate from C. Erba, (Milan, Italy) atractyloside was a kind gift from Prof. R. SANTI, University of Padova (Padova, Italy), and propionylcarnitine from Italseber (Milan, Italy).

RESULTS

In the presence of dinitrophenol and malonate, α-oxobutyrate was readily oxidized by isolated kidney mitochondria. The O_2 uptake was not affected by the addition of ATP, and attractyloside, nor by addition of CoA and carnitine, whereas the O_2 concumption was completely blocked by arsenite (Fig. 1A). It is conceivable that under these conditions (malonate-inhibited system) only the initial one-step oxidative decarboxylation of α-oxobutyrate occurs.

On the other hand, when malonate was omitted the oxidation of α-oxobutyrate was stimulated by externally added ATP. Atractyloside abolished the stimulatory effect of ATP and the combined addition of CoA and carnitine removed the in-

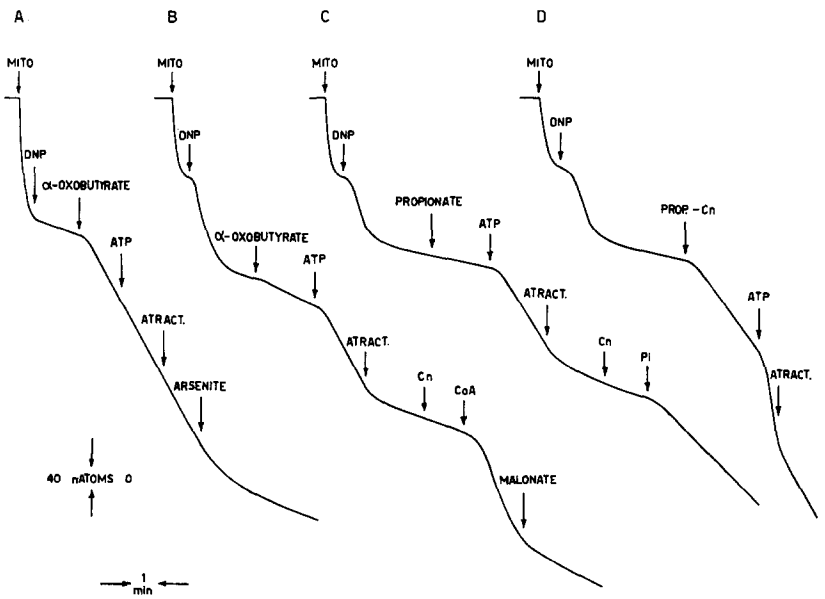

Fig. 1. O_2 uptake in the presence of α-oxobutyrate, propionate and propionylcarnitine by kidney mitochondria. A. The incubation system contained 13 mM Tris–HCl (pH 7.4), 30 mM NaCl, 6 mM $MgCl_2$, 10 mM malonate, 58 mM KCl, in a final volume of 1.8 ml. At the points indicated by arrows, 6 mg of mitochondrial protein, 0.1 μmole of dinitrophenol (DNP), 2.5 μmoles of α-oxobutyrate, 2 μmoles of ATP, 0.5 μmole of atractyloside, 4 μmoles of arsenite were added. B, C and D. The incubation medium as in A, but without malonate, *plus* 5 mM $NaHCO_3$. At the points indicated by arrows 4 mg mitochondrial protein, 0.1 μmole of DNP, 2.5 μmoles of α-oxobutyrate, 2.5 μmoles of propionate, 2.5 μmoles of propionylcarnitine, 2 μmoles of ATP, 0.5 μmole of atractyloside, 4 μmoles of carnitine, 0.1 μmole of CoA, 16 μmoles of P_i, 10 μmoles of malonate, were added.

hibitory effect of atractyloside. The addition of malonate strongly reduced O_2 uptake (Fig. 1B). It is important to outline that these results have been obtained in medium free of added P_i.

Under the same conditions the oxidation of propionate was completely dependent on added ATP and completely inhibited by atractyloside. Moreover the combined addition of CoA *plus* carnitine did not remove, nor overcome, the inhibitory effect of atractyloside, unless P_i was also added. In the presence of added P_i the addition of CoA was no longer necessary to remove the inhibition induced by atractyloside (Fig. 1C).

Propionylcarnitine was oxidized by kidney mitochondria with the same feature described for α-oxobutyrate, but at a faster rate than either α-oxobutyrate or propionate (Fig. 1D). In the presence of atractyloside the oxidation of propionylcarnitine was restored, as expected, by addition of CoA. ATP was also required, evidently because the oxidation of propionylcarnitine through propionyl-CoA[12] can be expected to involve a carboxylation step[13]. As in the case of α-oxobutyrate addition of P_i is not necessary for removing the inhibition by atractyloside. Therefore the need of P_i in a system inhibited by atractyloside seems to be peculiar for the activation processes[14] to which propionate, as well as fatty acids in general, must undergo in order to be oxidized, but not for the successive carboxylative reaction which is common to propionate, α-oxobutyrate and propionylcarnitine.

The relative rate of O_2 uptake of propionate, α-oxobutyrate and propionylcarnitine, expressed in natoms per 10 mg mitochondrial protein per min, were respectively: 91, 132 and 205.

The oxidation of α-oxobutyrate by liver mitochondria has properties similar to those shown by kidney mitochondria, with one significant difference: the oxidative decarboxylation, *i.e.* the oxidation in the presence of dinitrophenol *plus* malonate, was stimulated by carnitine. The stimulation by added carnitine was also evident in experiments over longer time intervals (in Warburg apparatus) which, moreover, showed a net disappearance of α-oxobutyrate (Table I). The lack of a close proportionality between O_2 uptake, CO_2 evolution and α-oxobutyrate disappearance can be

TABLE I

OXIDATION OF α-OXOBUTYRATE BY LIVER MITOCHONDRIA

O_2 uptake, CO_2 evolution and α-oxobutyrate disappearance per 10 mg mitochondrial protein per min. Incubation system contained: 13 mM Tris–HCl (pH 7.4), 30 mM NaCl, 58 mM KCl, 6 mM $MgCl_2$, 0.1 mM dinitrophenol and 10 mM α-oxobutyrate. Temp., 25°. Incubation time, 25 min. Analogous results have been obtained also with kidney mitochondria.

	O_2 uptake (natoms)	*CO_2 evolved (nmoles)*	*α-Oxobutyrate disappeared (nmoles)*
10 mM malonate	43	20	19
10 mM malonate + 2 mM ATP	48	21	20
10 mM malonate + 0.5 mM atractyloside	42	17	19
10 mM malonate + 4 mM carnitine	56	30	34
2 mM ATP	132	57	41
2 mM ATP + 0.5 mM atractyloside	44	20	17
2 mM ATP + 4 mM carnitine	215	66	64

explained considering that α-oxobutyrate gives rise ultimately to oxaloacetate, which is known to activate, or to accelerate, the oxidation of endogenous fatty acids[15] which certainly are responsible for the extra O_2 uptake, observed in the absence of malonate.

Addition of bicarbonate to the medium significantly increased (about 30 %) the O_2 uptake in the presence of α-oxobutyrate, propionylcarnitine and propionate.

DISCUSSION

Present results provide indirect but very consistent evidence that α-oxobutyrate oxidation does not pass through the formation of propionate, as one can argue from the results of KINNORY, TAKEDA AND GREENBERG[3], but through propionyl-CoA. In fact α-oxobutyrate is much more actively oxidized than propionate.

That this preferential oxidation is not merely due to a difference in permeability towards mitochondrial membranes is proved by the observation that in the presence of atractyloside the complete oxidation of propionate, unlike that of α-oxobutyrate, requires added P_i. Assuming that the inner membrane is impermeable to CoA and to CoA thioesters and that the atractyloside inhibits the exchange of the adenine nucleotides across the inner membrane[16,17], the data presented in this paper are consistent with the following proposals (see Fig. 2).

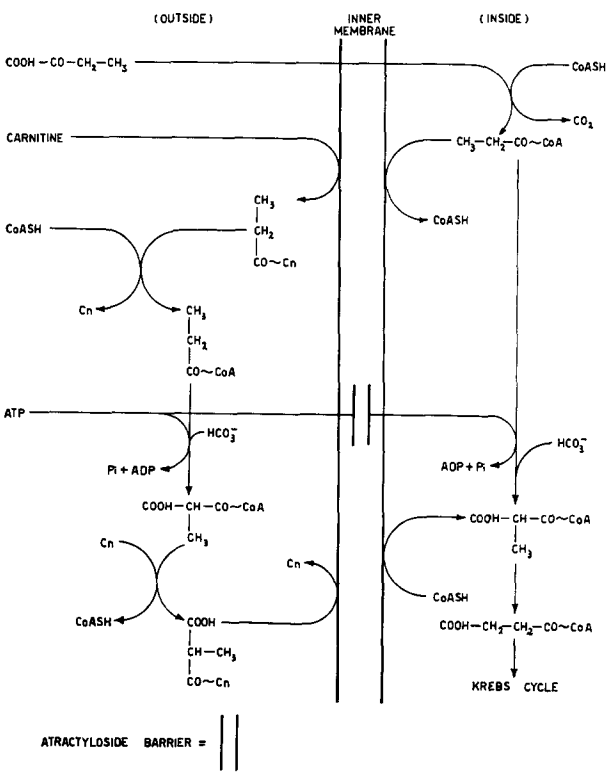

Fig. 2. Proposed scheme for mitochondrial oxidation of α-oxobutyrate.

The initial step of oxidative decarboxylation of α-oxobutyrate occurs internally to the atractyloside barrier, using endogenous CoA, which is available in sufficient amount in kidney mitochondria. The stimulatory effect of carnitine observed in liver mitochondria might be related to the fact that carnitine allows stoichiometric availability of free CoA for the propionyl-CoA formation[18].

In the presence of dinitrophenol added ATP is needed for the second step, that is, for the carboxylation of propionyl-CoA to methylmalonyl-CoA[19]. Atractyloside inhibits the carboxylative reaction by blocking the access of added ATP to the internal compartment. Therefore it can be concluded that the carboxylative reaction is operative inside the atractyloside barrier.

However, the combined addition of CoA and carnitine restores the activating action in the atractyloside-inhibited system. This observation supports the conclusion that the carboxylation of propionyl-CoA can also occur in an "external" compartment of mitochondria, *i.e.* outside the atractyloside-sensitive barrier (inner membrane)[17] as schematically drawn in Fig. 2.

Methylmalonyl-CoA, upon conversion into methylmalonylcarnitine could penetrate into the inner compartment where it is reconverted into methylmalonyl-CoA and isomerized to succinyl-CoA.

Alternatively methylmalonyl-CoA could be isomerized to succinyl-CoA outside the atractyloside barrier, and succinate, as such or in the form of succinylcarnitine, traverses the "barrier" and is then oxidized to completion.

Propionate can be activated to propionyl-CoA outside the atractyloside barrier, but P_i is then required. Propionate oxidation by mitochondria exhibits the same features as those of long chain fatty acids[14,20–23] and likewise its activation can occur both inside and outside the atractyloside barrier[14,23].

In conclusion, the results reported in the present paper show that the initial oxidative decarboxylation of α-oxobutyrate occurs inside the atractyloside barrier of mitochondria, while the carboxylation step propionyl-CoA → methylmalonyl-CoA, appears to occur, like fatty acid activation[14,23] either inside or outside the barrier.

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