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PERMEABILITY OF ISOLATED MITOCHONDRIA TO OXALOACETATE

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SUMMARY

1. The transport of oxaloacetate in mitochondria from several sources has been studied.

2. Oxaloacetate is rapidly transported across the membrane of isolated rat-liver mitochondria. The V is 80–270 nmoles/min per mg protein at 30 °C. The K_m for oxaloacetate is 80–130 μM.

3. Oxaloacetate can exchange either for another dicarboxylate ion or for phosphate. This exchange is inhibited by butylmalonate. It is concluded that oxaloacetate is transported *via* the dicarboxylate translocator.

4. A rapid rate of transport is found only in tightly coupled mitochondria. The K_m for oxaloacetate is independent of the coupling of the mitochondria.

5. A rapid transport of oxaloacetate is found in mitochondria from rat liver and guinea-pig liver, but not in those from rabbit or pigeon liver or from rat heart.

INTRODUCTION

Although it has generally been accepted in the past that mitochondria are impermeable to oxaloacetate[1], several recent reports[2–7] have indicated that this may not be the case in intact mitochondria isolated from rat liver.

Haslam and Krebs[2] and Haslam and Griffiths[3] used the rate of oxidation of intramitochondrial nicotinamide nucleotides by added oxaloacetate (see also ref. 8) as a measure of the permeability of the mitochondria for the oxo acid, on the assumption that the activity of mitochondrial malate dehydrogenase is not the rate-limiting step[9]. They observed a rate of oxidation of NAD(P)H of 1–4 nmoles/per min per mg protein (V, about 15 nmoles/min per mg protein; K_m for oxaloacetate, about 30–40 μM).

Kunz and co-workers[5,6] applying essentially the same technique, found a V for oxaloacetate transport of 60–80 nmoles/min per mg protein (K_m for oxaloacetate, 0.2–0.5 mM). Both groups suggested that oxaloacteate translocation is of no physiological importance because of the low intracellular concentration of oxaloacetate in comparison with the K_m for transport of the substrate. An even higher K_m for oxaloacetate (about 1 mM) was estimated by Robinson and Chappell[7].

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We have reinvestigated the problem of the permeability of mitochondria to oxaloacetate, and have studied the mechanism and rate of transport by following the reactions during the first few seconds after addition of the substrate. It was found that oxaloacetate is transported rapidly (V, 80–270 nmoles/min per mg protein) in mitochondria from rat and guinea-pig liver, but not in those from rat heart, rabbit liver, or pigeon liver. The transport of oxaloacetate is mediated by the dicarboxylate translocator[9,10].

MATERIALS AND METHODS

Mitochondria

Mitochondria from rat liver, guinea-pig liver and rabbit liver were prepared according to the method of Hogeboom[11] as described by Myers and Slater[12]. In the case of guinea-pig liver and rabbit liver, the mitochondria were washed twice with 0.25 M sucrose. Rat-heart mitochondria were prepared according to the method of Holton *et al.*[13].

Pigeon-liver mitochondria were prepared as follows. The livers from 1 or 2 pigeons were finely cut in ice-cold 0.25 M sucrose and rinsed with sucrose until no yellow colour due to bile could be seen. The liver mince was homogenized in ice-cold 0.25 M sucrose (total volume, 100 ml), and the homogenate was centrifuged for 10 min at $27\,500 \times g$. The supernatant was discarded and the pellet was rehomogenized in ice-cold 0.25 M sucrose. Finally the mitochondria were isolated by differential centrifugation as described for rat-liver mitochondria[12].

Standard reaction medium

A standard reaction medium containing the following components was used: 250 mM sucrose, 15 mM Tris–HCl, 1 mM EDTA and 2 μg rotenone. The final pH was 7.4. The final incubation volume was 1 ml. The temperature was 30 °C.

Incubations

Incubations were carried out in small thermostatted vessels with continuous stirring as described by Van Dam[15]. Intramitochondrial NAD(P) was reduced by preincubation of the mitochondria in the standard reaction medium for 2 min. The reactions were started by the rapid addition of oxaloacetate and stopped at the times indicated in the figures by the rapid addition of 0.10 ml 35% $HClO_4$. The acid extracts were neutralized with 1 M KOH and kept in the cold for 30 min, after which $KClO_4$ was removed by centrifugation.

Loading of the mitochondria with anions

This was performed as follows. The mitochondrial preparation (100–150 mg protein) was incubated for 5 min at 0 °C in a medium (10 ml) containing 250 mM sucrose, 10 mM Tris–HCl (pH 7.5) and 100 μg rotenone (to ensure a high reduction level of endogenous NAD(P)). Subsequently 5–10 mM of the potassium salt of the desired anion was added. After 5 min further incubation the mitochondria were washed twice with 0.25 M sucrose and finally suspended in 0.25 M sucrose.

Determinations

NAD^+ and $NADP^+$ were determined enzymically in the neutralized acid extracts, and *NADH and NADPH* in neutralized alkaline extracts, according to Klingenberg[14], using the Aminco-Chance dual-wavelength spectrophotometer (wavelength pair 350–375 nm). Alkaline extracts were obtained by stopping the reaction with 0.5 ml 1 M KOH in 96% ethanol, and neutralized with 0.5 ml of a mixture containing 0.5 M triethanolamine-HCl, 0.4 M KH_2PO_4 and 0.1 M K_2HPO_4.

Malate was determined with citrate synthase, malate dehydrogenase, NAD^+ and acetyl-CoA[16].

α-Oxoglutarate was determined according to the method of Slater and Holton[17] with NADH, NH_4Cl and glutamate dehydrogenase.

Inorganic phosphate was determined according to the method of Wahler and Wollenberger[18].

Protein was determined by the biuret method according to Cleland and Slater[19], using egg albumin as standard.

Enzymes

Enzymes used in the assays of metabolites were obtained from Boehringer und Söhne, Mannheim, Germany.

RESULTS AND DISCUSSION

Kinetics of oxaloacetate uptake

The rate of uptake of oxaloacetate was estimated indirectly by measuring the rate of oxidation of intramitochondrial nicotinamide nucleotides upon addition of oxaloacetate to intact mitochondria[2,3,5,6].

Fig. 1 shows the kinetics of the oxidation of intramitochondrial NADH and NADPH by oxaloacetate added at two concentrations. It should be emphasized that oxidized nicotinamide nucleotides were estimated enzymically after acid extraction of the mitochondria at the times indicated. It is clear from Fig. 1 that at both substrate concentrations maximal oxidation of NADH occurred within about 10 s. The rates of NADH oxidation during the first few seconds after addition of 20 and 500 μM oxaloacetate were 31 and 93 nmoles/min per mg protein, respectively. After a short lag period of about 5 s, NADPH was oxidized at a constant rate of 1.4 or 3.0 nmoles/min per mg protein with 20 or 500 μM oxaloacetate, respectively.

These results show that the initial rate of oxaloacetate uptake, as measured by NAD(P)H oxidation, can only be measured accurately in the first few seconds of the reaction. Essentially similar results were obtained when NAD(P)H oxidation was followed spectrophotometrically in the reaction mixture, using a dual-wavelength spectrophotometer with rapid-response electronics. An initial rapid phase of NAD(P)H oxidation was followed after about 2 s by a slow phase (not shown). Fig. 1 also shows that after addition of 500 μM oxaloacetate, NAD reached a higher oxidation level than after addition of 20 μM oxaloacetate.

Control experiments showed that in the first 10–20 s of the reaction, $NAD(P)^+$ formation equalled malate production and oxaloacetate disappearance. This is illustrated in Table I. However, when longer times were used, malate formation

was greater than net NAD(P)H oxidation, due to reduction of nicotinamide nucleotides by endogenous substrates (not shown).

In Fig. 2 a Lineweaver–Burk plot of the rate of uptake of oxaloacetate as a function of the concentration of oxaloacetate is presented. In this experiment

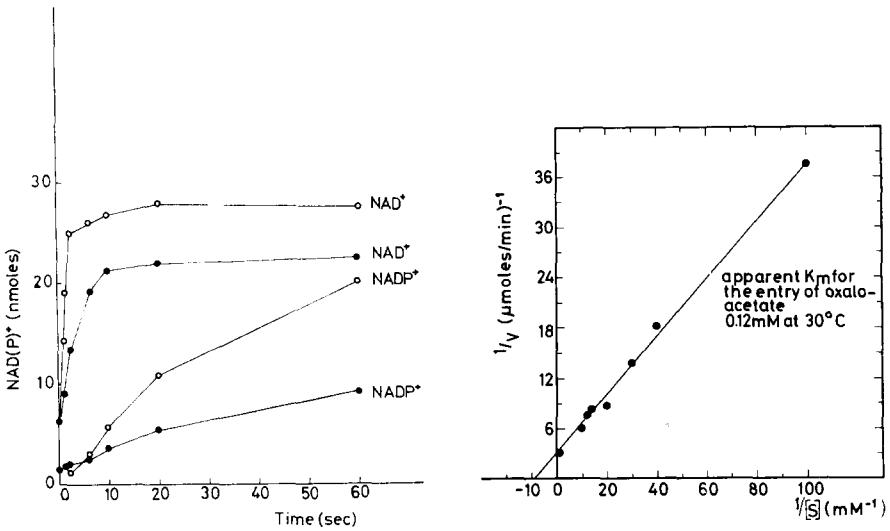

Fig. 1. Oxidation of intramitochondrial NADH and NADPH by added oxaloacetate. Rat-liver mitochondria (5.8 mg protein) were preincubated at 30 °C for 2 min in a medium containing the standard components. After addition of 20 μM (●—●) or 500 μM (○—○) oxaloacetate, the reaction was carried out in parallel incubations for the times indicated. NAD^+ and $NADP^+$ were determined in the neutralized acid extracts.

Fig. 2. Lineweaver–Burk plot of the rate of oxidation of the intramitochondrial NADH of mitochondria by added oxaloacetate, at 30 °C. In this experiment the initial rate of oxidation of the intramitochondrial NADH was followed with a double-beam spectrophotometer with rapid-response electronics. The reaction mixture (final volume, 1 ml) contained 1.7 mg mitochondrial protein. Apparent K_m, 0.12 mM; apparent V, 180 nmoles/min per mg protein.

TABLE I

STOICHEOMETRY OF THE DISAPPEARANCE OF OXALOACETATE AND NADH AND THE FORMATION OF NAD^+ AND MALATE IN RAT-LIVER MITOCHONDRIA ON THE ADDITION OF OXALOACETATE

The incubations were carried out with freshly-prepared mitochondria as described in Fig. 1. The initial rates are given.

Expt	*Additions*	*−Δ oxaloacetate*	*Δ malate*	*−ΔNAD(P)H*	*ΔNAD(P)⁺*
		nmoles/min per mg protein			
1	Oxaloacetate (35 μM)	32	29	28	26
2	Oxaloacetate (500 μM)	—	238	191	214
3	Oxaloacetate (300 μM) +butylmalonate (5 mM)	—	9.5	4.0	7.5

a K_m of 0.12 mM was found, and the V was 180 nmoles/min per mg protein. In other experiments V values of 80–270 nmoles/min per mg protein were obtained, the highest values being found in mitochondrial preparations with the highest respiratory control indices (see Fig. 3). On the other hand, the K_m values for oxaloacetate were relatively constant (0.08–0.13 mM) and independent of the preparation. Our values for the rate of oxaloacetate uptake are higher than those reported by Haslam *et al.*[2,3] (1–4 nmoles/min per mg protein at 30 °C) or by Kunz and co-workers[5,6] (60–80 nmoles/min per mg protein at 22 °C). The low values reported by these groups[2,3], may have been due to the fact that the initial velocity of the reaction was not measured.

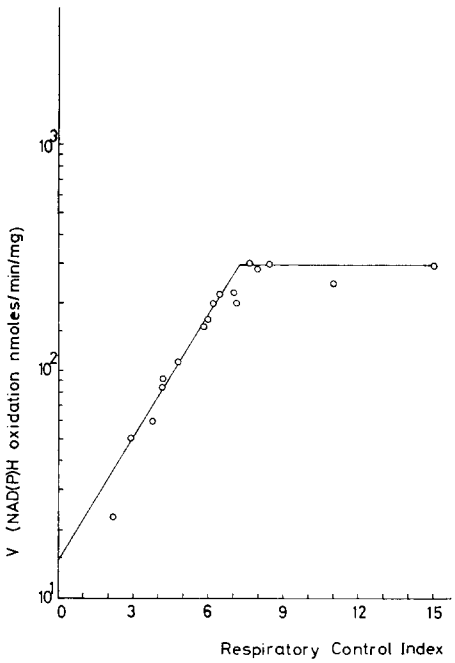

Fig. 3. Relationship between the respiratory control index and the initial intramitochondrial NAD(P)H oxidation, by added oxaloacetate at 30 °C. The concentration of oxaloacetate was 0.5 mM. Experiments in which the amount of NAD^+ at time zero was greater than 1 nmole/mg protein are not included.

Mechanism of oxaloacetate uptake

Fig. 4A shows that butylmalonate, the inhibitor of the dicarboxylate anion translocator[20,21], inhibits the oxidation of intramitochondrial NAD(P)H by oxaloacetate substantially. In this experiment freshly prepared mitochondria were used. In the experiments of Fig. 4B, 4C and 4D, the mitochondria were preloaded with various anions. The rate of oxaloacetate uptake in mitochondria loaded with phosphate (Fig. 4B) or malonate (Fig. 4C) was of the same order of magnitude as that of untreated mitochondria. In both cases the exchange was sensitive to butyl-malonate.

These results suggest that oxaloacetate is transported across the mitochondrial membrane *via* the dicarboxylate translocator in exchange either for another dicarboxylic acid anion or for phosphate. In agreement with this conclusion, it was found that phosphate or dicarboxylate anions like succinate or malonate competitively inhibit oxaloacetate transport (not shown; see also refs 3, 5, 6).

Fig. 4D shows that when mitochondria were preloaded with α-oxoglutarate, addition of oxaloacetate led to oxidation of NAD(P)H. Unexpectedly, it was found

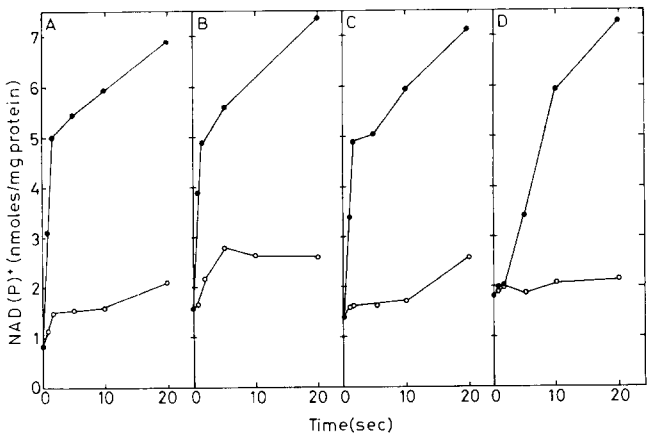

Fig. 4. Effect of butylmalonate on the uptake of oxaloacetate by untreated mitochondria or mitochondria preloaded with different anions. The whole mitochondrial suspension was loaded with phosphate (B), malonate (C), or α-oxoglutarate (D), by incubation for 5 min at 0 °C in a medium (10 ml) containing 0.25 M sucrose, 10 mM Tris–HCl (pH 7.5) and 10 mM of the salt of the anion. The preparation was diluted with 50 ml ice-cold sucrose (0.25 M, 50 ml) and centrifuged. The mitochondrial pellet was washed twice with 0.25 M sucrose, and finally the mitochondria were taken up in about 2 ml 0.25 M sucrose. The mitochondria were incubated as described in the legend to Fig. 1. (A) Untreated mitochondria. (B) Phosphate-loaded mitochondria; 114 nmoles phosphate per mg protein were present. (C) Malonate-loaded mitochondria. (D) α-Oxoglutarate-loaded mitochondria; 10.5 nmoles α-oxoglutarate per mg protein were present. ○—○, 5 mM butylmalonate; ●—●, no further additions.

TABLE II

EFFECT OF BUTYLMALONATE ON THE INITIAL VELOCITY OF OXIDATION OF INTRAMITOCHONDRIAL NADH BY ADDED OXALOACETATE

The incubations were carried out with freshly prepared mitochondria as described in Fig. 1. The inhibitor was present during the preincubation. The oxaloacetate concentration was 0.5 mM.

Butylmalonate (mM)	*ΔNAD^+ (nmoles/min per mg protein)*	*Inhibition (%)*
0	172	—
0.5	1.8	98.5
1	1.6	99
5	2.1	98

that this oxidation was inhibited by butylmalonate. Although a separate α-oxoglutarate translocator exists which exchanges α-oxoglutarate for a dicarboxylate anion like malate or malonate, this exchange is relatively insensitive to butylmalonate[21]. Thus although the possibility existed that oxaloacetate might exchange for α-oxoglutarate on the α-oxoglutarate translocator, this exchange, too, would be expected to be insensitive to butylmalonate. The experiment of Table II shows that almost complete inhibition of oxaloacetate transport was obtained when the butylmalonate:oxaloacetate ratio was as low as 1:1. The α-oxoglutarate–malate exchange is not inhibited by butylmalonate even when the butylmalonate: malate ratio is 50 (Table III).

TABLE III

EFFECT OF BUTYLMALONATE ON THE MALATE–α–OXOGLUTARATE EXCHANGE IN α-OXOGLUTARATE-LOADED MITOCHONDRIA

The α-oxoglutarate-loaded mitochondria (about 3 mg protein) were incubated at room temperature for 45 s in a medium (final volume 0.5 ml) containing 20 mM KCl, 1 mM EDTA, 5 mM $MgCl_2$, 50 mM Tris–HCl, 2 mM arsenite, 2 μg rotenone and the additions as indicated. Final pH, 7.5. The mitochondria were separated from the medium by silicone oil centrifugation–filtration as described by Meijer[21]. α-Oxoglutarate was determined in the pellets and in the supernatants.

Additions	*α-Oxoglutarate (nmoles)*	
	In	*Out*
None	85.5	16.0
Malate (1 mM)	10.0	78.5
Malate + butylmalonate (5 mM)	14.0	81.0
Malate + butylmalonate (50 mM)	10.0	69.5

A possible explanation of these results is that in α-oxoglutarate-loaded mitochondria, oxaloacetate is not exchanged for α-oxoglutarate, but for a small amount of phosphate or malate still present in these mitochondria. Inside the mitochondria this oxaloacetate would be reduced to malate, which in turn, could be exchanged again for oxaloacetate. Furthermore, this extramitochondrial malate would be taken up again in exchange for α-oxoglutarate. Thus the intramitochondrial pool of malate would increase rapidly. In a control experiment, α-oxoglutarate was measured. It was found that addition of oxaloacetate led to an extrusion of α-oxoglutarate from the mitochondria (3.5 nmoles/mg protein in 1 min), and that this extrusion was inhibited by butylmalonate. Some support for this coupling of two translocator activities is found in the fact that the maximal rate of oxaloacetate uptake in the case of α-oxoglutarate-loaded mitochondria is reached only after a short lag period (Fig. 4D), whereas in the other cases (Figs 4A, B and C), no lag was observed. The lag was more pronounced with mitochondria that contained more α-oxoglutarate after the loading procedure (not shown).

Oxaloacetate permeability in mitochondria from different tissues

Fig. 5 shows the results of experiments on oxaloacetate transport, measured as NAD(P)H oxidation, in mitochondria from various sources. Rat-liver mitochondria show the highest rate of NADH oxidation upon addition of oxaloacetate.

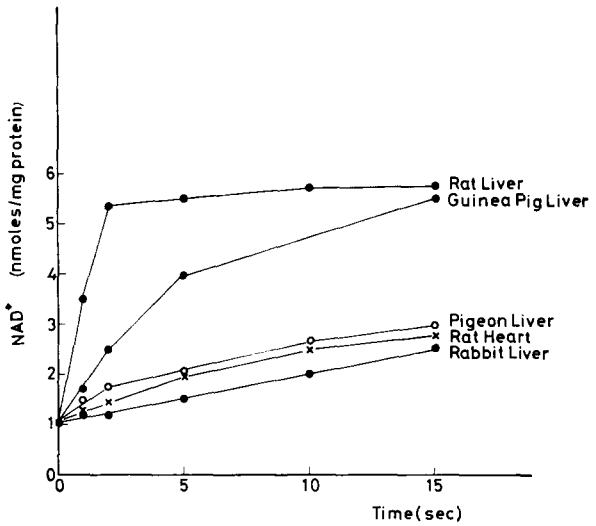

Fig. 5. Oxidation of intramitochondrial NADH by added oxaloacetate (0.5 mM) in various types of mitochondria, at 30 °C. The experiments were performed as described in Fig. 1.

Mitochondria from rat heart, rabbit liver or pigeon liver, on the other hand, are virtually impermeable to this anion. No oxaloacetate transport could be demonstrated in these mitochondria even when the concentration of the anion was raised to 10 mM. Guinea-pig liver mitochondria possess an intermediate permeability to oxaloacetate. These striking differences suggest that there might be a functional role for the transport of oxaloacetate across the mitochondrial membrane in rat-liver and guinea-pig-liver mitochondria. It is possible that the entry of oxaloacetate, in combination with an extrusion of malate from the mitochondria, constitutes a malate–oxaloacetate cycle for the transfer of reducing equivalents from the mitochondria to the cytosol, as suggested by Delbrück[23] and Krebs[24]. The extremely low oxaloacetate permeability in rat-heart mitochondria is not in contradiction with this suggestion, because in rat heart *in vivo*, transfer of reducing equivalents from the mitochondrion to the cytosol would not be expected to be important. However, the question remains unexplained of why the malate–oxaloacetate cycle should not function in rabbit or pigeon liver.

Another possibility is that oxaloacetate transport plays a role in providing the cytosol with precursors for gluconeogenesis. It is striking that rat liver, in which phosphoenolpyruvate carboxylase (GTP:oxaloacetate carboxylyase (transphosphorylating), EC 4.1.1.32) is localized in the cytosol[25], is permeable to oxaloacetate, whereas rabbit and pigeon liver, producing phosphoenolpyruvate intramitochondrially[26], are not permeable to oxaloacetate. In guinea-pig liver, where

phosphopyruvate carboxylase is present both in the cytosol and in the mitochondria[25], the oxaloacetate permeability of the mitochondria is intermediate.

Whatever the function of oxaloacetate transport may be, it is clear that in different types of mitochondria the dicarboxylate anion translocator has different properties with respect to the translocation of oxaloacetate. Possibly two (or more) different translocators for dicarboxylate anions exist, one (or more) of which is absent in mitochondria from certain tissues.

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