THE INHIBITION OF 2-OXOGLUTARATE ENTRY INTO RAT LIVER MITOCHONDRIA BY L-ASPARTATE

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1. Introduction

The transport of 2-oxoglutarate across the inner membrane of rat liver mitochondria is mediated by a specific carrier system which is activated by phosphate and L-malate [1–4]. 2-n-Butyl malonate inhibits this carrier by competition with the malate activator [5].

In this communication, evidence is presented that the entry of oxoglutarate into mitochondria is specifically inhibited by L-aspartate. The inhibition is competitive with respect to oxoglutarate (K_i = 0.15 mM), and non-competitive with respect to L-malate or malonate. Externally added aspartate does not appear to influence oxoglutarate efflux from mitochondria. It is suggested that in vivo the oxoglutarate carrier is effectively unidirectional.

2. Methods

Rat liver mitochondria were prepared as described by Chappell and Hansford [6]. Changes in the redox state of the intramitochondrial nicotinamide nucleotides were monitored by double-beam spectrophotometry at 340–373 mμ. Oxoglutarate and L-aspartate were assayed enzymatically.

3. Results

The rate of reduction of intramitochondrial NAD(P) by externally added oxoglutarate together with malonate (to activate oxoglutarate entry) (fig. 1(A)) was considerably slower if L-aspartate was added before the oxoglutarate (fig. 1(B)). Similar results were obtained using L-malate as activator. In this type of experiment, it was essential to prevent the possibility of transamination between oxoglutarate and aspartate. This was achieved by prior addition of 1 mM hydroxylamine or 1 mM KCN, either of which virtually completely inhibited the mitochondrial aspartate aminotransferase. Control experiments with ultrasonically treated mitochondria showed that aspartate in the presence of KCN did not significantly inhibit the activity of oxoglutarate dehydrogenase.

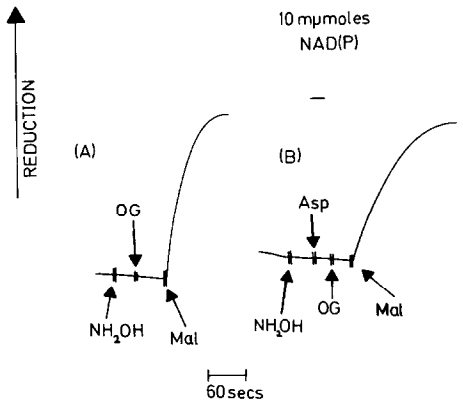

Fig. 1. Effect of L-aspartate on the reduction of intramitochondrial NAD(P) by oxoglutarate in the presence of malonate. Rat liver mitochondria (6 mg) were suspended in a medium containing 120 mM-KCl, 20 mM-tris chloride and 5 mM-tris phosphate, pH 7.4 in the cuvette of a double-beam spectrophotometer. The total volume was 2.5 ml. 1μM FCCP was added and after a 3 min incubation at 30°C, this was followed by 0.5 μg antimycin (not shown). Further additions were hydroxylamine (1 mM), oxoglutarate (0.5 mM), aspartate (1 mM) and malonate (1 mM). The recorder was reset after the oxoglutarate addition which caused a non-specific absorbancy change.

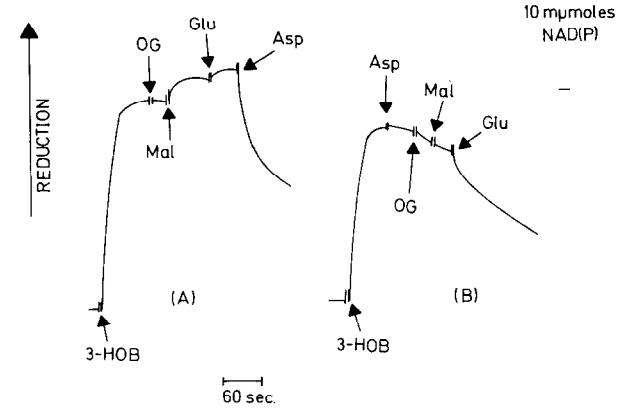

Fig. 2. Effect of the order of addition of reagents on the oxidation of intramitochondrial NAD(P)H. The mitochondria were incubated with FCCP as in fig. 1, and antimycin was added after 3 minutes. Further additions were 3-hydroxybutyrate (2 mM), oxoglutarate (1 mM), malonate (1 mM), glutamate (1 mM), aspartate (1 mM). The temperature was 30°C.

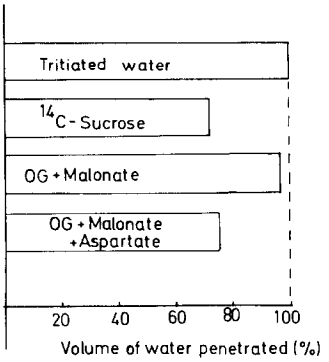

Fig. 3. Penetration of the water of mitochondrial pellets by oxoglutarate in the presence of malonate, and the inhibitory effect of aspartate. Mitochondria (20 mg) were incubated for 5 min at 20°C in a medium containing 120 mM-KCl, 20 mM-tris chloride, 5 mM-oxoglutarate, 5 mM-malonate, 5 mM phosphate, 1 mM hydroxylamine, 1 mM arsenite, antimycin (0.5 μg) and rotenone (1 μg) in a total volume of 3 ml pH 7.4. The incubation medium also contained ^{14}C-labelled sucrose and tritiated water and in some experiments 5 mM-aspartate was also present. The mitochondria were sedimented by centrifugation, and oxoglutarate, ^{14}C-labelled sucrose and tritiated water were determined in the pellet and supernatant after deproteinisation with perchloric acid. For details, see Chappell et al. [3].

Fig. 2(A) shows that when the intramitochondrial nucleotides were reduced by the addition of 3-hydroxybutyrate, the addition of oxoglutarate together with malonate followed by glutamate (to activate aspartate entry [7]) and aspartate led to a rapid reoxidation of NADH. This oxidation was due to the production of oxaloacetate by the transamination of oxoglutarate and aspartate. Fig. 2(B) shows the same experiment except that aspartate was added before oxoglutarate. The rate of NADH oxidation was slower, suggesting that the access of oxoglutarate to the transaminase was inhibited by aspartate. Glutamate entry was not inhibited by aspartate.

Similarly, it was shown that the oxidation of NAD(P)H (in intact mitochondria) on the addition of oxoglutarate together with malonate and NH_4^+, due to the action of glutamate dehydrogenase, was inhibited by aspartate. Glutamate dehydrogenase activity of ultrasonically or triton-treated mitochondria was not affected by aspartate. Again hydroxylamine was present to prevent transamination. Thus the interaction of oxoglutarate with the intramitochondrial enzymes aspartate aminotransferase, oxoglutarate and glutamate dehydrogenase was inhibited by the prior addition of aspartate, suggesting that aspartate was inhibiting oxoglutarate entry. The inhibition was specific to the oxoglutarate carrier, in that the entry of citrate, glutamate or malate was not inhibited by aspartate.

In experiments similar to those in fig. 1 it appeared that the extent of the inhibition depended on the relative concentrations of oxoglutarate and aspartate. Plots of the reciprocal of the rate of NAD(P) reduction against inhibitor (aspartate) concentration showed that the inhibition was competitive with respect to oxoglutarate (K_i = 0.15 mM), and non-competitive with respect to malate or malonate.

The intramitochondrial volume available to oxoglutarate was determined as described by Chappell [4], using ^{14}C-labelled sucrose and ^3H_2O as markers. In the absence of aspartate, oxoglutarate penetrated the mitochondria to the same extent as ^3H_2O (see also [3]). However in the presence of aspartate the extent of oxoglutarate penetration was reduced to a level only slightly above that of ^{14}C-labelled sucrose (fig. 3).

The inhibition of oxoglutarate entry by aspartate could be demonstrated also by the ammonium swelling technique [2]. Thus the rate of swelling of mitochondria in a medium containing 50 mM ammonium oxoglutarate and 70 mM NH_4Cl was much faster than that in 50 mM NH_4^+ oxoglutarate and 50 mM NH_4 as-

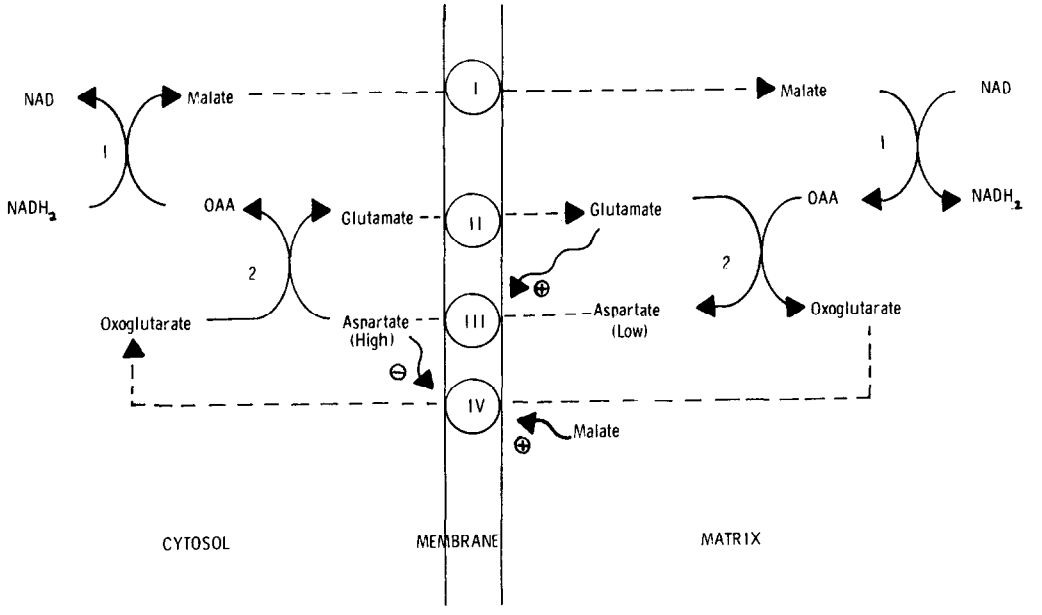

Fig. 4. Postulated "shuttle" for the transfer of reducing equivalents across the mitochondrial membrane [12,3,4]. Activating effects of compounds on carriers are indicated by wavy arrows with a positive sign, and inhibition by wavy arrows with a negative sign.

I malate transporter
II glutamate transporter
III aspartate transporter, glutamate activated
IV oxoglutarate transporter, malate activated, entry of oxoglutarate inhibited by aspartate.

1: malate dehydrogenase
2: aspartate aminotransferase
OAA: oxaloacetate.

partate (Cl^- is a non-penetrant). Swelling was initiated by the addition of 2 mM malate, and 2 mM NH_4^+ phosphate was present initially.

When glutamate is oxidised in the presence of arsenite and excess ADP and phosphate, oxoglutarate accumulates in the mitochondria and after 3–5 minutes the rate of oxoglutarate production is equal to the rate of oxoglutarate efflux from the mitochondria [9]. In such experiments it was found that the addition of 5 mM aspartate to a mitochondrial suspension oxidising glutamate in the presence of arsenite did not inhibit the rate of oxoglutarate production when malonate was present, in the presence or absence of a transaminase inhibitor. In further similar experiments, the distribution of oxoglutarate with time was measured in the presence and absence of aspartate. No significant difference was found. Thus externally added aspartate did not influence oxoglutarate efflux, suggesting that oxoglutarate and aspartate must be on the same side of the membrane for inhibition to occur.

Under no metabolic conditions investigated could a significant intramitochondrial aspartate concentration be detected. For example, when mitochondria oxidise malate in the presence of glutamate, the oxaloacetate formed is transaminated with the glutamate to form oxoglutarate and aspartate. However, measurements of aspartate distribution showed that practically all the aspartate formed was outside the mitochondria. The very low concentrations of aspartate observed inside mitochondria indicate that oxoglutarate efflux could not be inhibited by aspartate.

The concentration of oxoglutarate in the normal rat liver is approximately 0.25 mM [10] with an apparent Km for entry of approximately 0.1 mM, and the intracellular concentration of aspartate is approximately 2 mM [11]. Thus it appears that *in vivo*, the carrier promotes oxoglutarate efflux much faster than its entry (if the intramitochondrial aspartate level is indeed low) i.e. the carrier is effectively unidirectional.

It has been suggested that the transfer of reducing

equivalents between the cytosol and the mitochondria occurs by the system shown in fig. 4 [12,3,4]. One of the problems with this system is to account for the considerable difference in redox state of the NAD in the two compartments (see [10]). In part, the energy-linked accumulation of malate (B.H.Robinson – unpublished observations), the observed exclusion of aspartate from the mitochondrion and the inhibition of oxoglutarate entry (but not efflux) by aspartate may be responsible for the relatively high degree of reduction of intramitochondrial NAD.

References

[1] A.J.Meijer and J.M.Tager, Biochem. J. 100 (1966) 79P.
[2] J.B.Chappell and K.N.Haarhoff, in: Biochemistry of Mitochondria, eds. E.C.Slater, Z. Kaniuga and L.Wojtozak (Academic Press, London, 1967) p. 75.
[3] J.B.Chappell, P.J.F.Henderson, J.D.McGivan and B.H. Robinson, in: The Interaction of Drugs and Subcellular Components in Animal Cells, ed. P.N.Cambell (Churchill, London, 1967) p. 71.
[4] J.B.Chappell, Brit. Med. Bull. 24 (1968) 150.
[5] B.H.Robinson and J.B.Chappell, Biochem. Biophys. Res. Commun. 28 (1967) 249.
[6] J.B.Chappell and R.G.Hansford, in: Subcellular Components – Preparation and Fractionation (Butterworths, London, 1969) p. 43.
[7] A.Azzi, J.B.Chappell and B.H.Robinson, Biochem. Biophys. Res. Commun. 29 (1967) 148.
[8] M.Dixon and E.C.Webb, Enzymes (Longmans, London, 1964) p. 315.
[9] S.Papa, E.J.de Haan, A.Francavilla, J.M.Tager and E. Quagliariello, Biochim. Biophys. Acta 143 (1967) 438.
[10] D.H.Williamson, P.Lund and H.A.Krebs, Biochem. J. 103 (1967) 514.
[11] J.R.Williamson, Adv. Enz. Reg. 5 (1967) 229.
[12] P.Borst, in: Funktionelle und Morphologische Organisation der Zelle (Springer-Verlag, Würzburg, 1963) p. 137.