PENETRATION OF THE MITOCHONDRIAL MEMBRANE BY GLUTAMATE AND ASPARTATE

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Received September 9, 1967

Evidence has been presented for the existence of systems located in mitochondrial membranes for the transport of phosphate (Chappell & Crofts, 1966), dicarboxylic acids (Chappell & Haarhoff, 1967; Robinson & Chappell, 1967), citrate, isocitrate and cis-aconitate (Chappell, 1964, 1966; Chappell & Haarhoff, 1967; Chappell, Henderson, McGivan & Robinson, 1967) and for oxoglutarate (Meijer & Tager, 1966; Chappell et al., 1967). In this paper results are presented which reveal the probable existence of two more carrier-systems, one for L-glutamate and one for L-aspartate. The latter carrier requires the presence of L-glutamate, or certain analogues of this amino acid, before it is able to act. The evidence for the glutamate carrier is, firstly, that the rate of reduction of intramitochondrial NAD(P) by glutamate has an optimum below pH 6, whereas with the free dehydrogenase the pH optimum is greater than 8. Secondly, certain analogues of glutamate, namely 3-hydroxyglutamate, 2-aminoadipate and threo-hydroxyaspartate inhibit competitively the reduction of intramitochondrial NAD(P) by glutamate, but have no or negligible effect on the activity of enzyme in mitochondrial extracts. The evidence for the aspartate carrier rests on the fact that aspartate is unable to react rapidly with mitochondrial aspartate aminotransferase until glutamate, or of the compounds tested hydroxyglutamate, aminoadipate or threo-hydroxyaspartate, are present.

METHODS AND MATERIALS. Mitochondria were prepared by conventional methods in a medium containing 0.25M-sucrose, 3.4mM-tris chloride and 1mM-ethyleneglycol- bis -(aminoethyl) tetracetate (EGTA), pH 7.4. The twice-washed mitochondria were stored at 0^{o} in a suspension containing 60-80 mg of mitochondrial protein/ml. Changes in the redox-state of intramitochondrial nicotinamide nucleotides were followed by fluorimetry (excitation with a quartz-iodine lamp with a 338 mμ interference filter (Baltzer); emitted light measured with a photomultiplier with a Kodak "Wratten" 2B filter) or by double-beam spectrophotometry at 340-373 mμ. Two spec-

trophotometers have been employed, the first using quartz-prism monochromators, the second interference filters. Both spectrophotometers gave quantitatively the same results.

L-Allo-3-hydroxyglutamate, L-2-aminoadipate, DL-erythro- and threo-hydroxyaspartates were obtained from Calbiochem Ltd., London.

RESULTS

Glutamate transport. The addition of L-glutamate to liver mitochondria resulted in a rapid and extensive reduction of intramitochondrial NAD(P). This was the case with mitochondria which had been incubated with ADP together with phosphate or with uncoupling agents. This procedure served to remove some of the endogenous substrates and under these conditions isocitrate or 2-oxoglutarate failed to cause extensive reduction of NAD(P) or get oxidized, unless L-malate or certain other dicarboxylic acids were added (see Chappell et al., 1967 and Fig. 2). It would appear therefore that liver mitochondria are freely permeable to glutamate. However, because glutamate is a relatively large charged molecule it would seem likely that some carrier mechanism must exist for its transport. Evidence that this is so is revealed by the following experiments.

The rate of reduction of intramitochondrial NAD(P) by glutamate was followed as a function of the pH of the suspending medium (Fig. 1). The optimum was below pH 6.5. It was not possible to follow rates below this pH value because the mitochondria aggregated, causing considerable interference with the spectrophotometric and fluorimetric assays. With mitochondria which had been sonicated and to which NAD had been added the pH-activity profile revealed that the optimum lay above pH 8. This experiment does not imply that a carrier-system is involved, since it is possible that when the glutamate dehydrogenase is bound to the mitochondrial structure it has a different pH optimum.

In an attempt to assess this, glutamate dehydrogenase activity was followed in intact and broken mitochondria in the reverse direction, i.e. on addition of oxoglutarate and NH_4^+. In this case the pH optimum lay on the alkaline side with both intact and broken mitochondria. This finding indicates that structural binding of the enzyme is not responsible for the difference in pH optimum of the dehydrogenase in intact mitochondria and in the free state.

That a carrier exists for the transport of glutamate across the mitochondrial membranes is indicated by the effects of certain analogues of glutamate on the rate of reduction of intramitochondrial NAD(P) by L-glutamate. Thus L-2-aminoadipate, L-allo-3-hydroxyglutamate and DL-

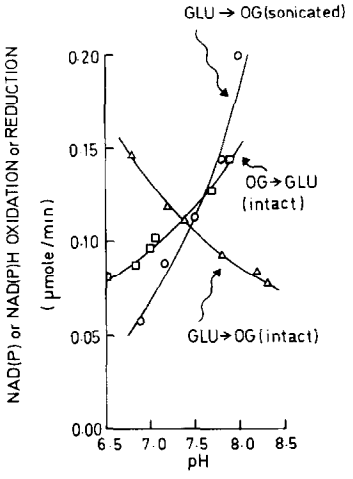

Fig. 1. The rate of reduction of NAD(P) by glutamate as a function of pH with intact and sonicated rat liver mitochondria. In the experiments with intact mitochondria labelled (△-△) the proceedure was as follows. 100μl of rat liver mitochondria (approximately 6 mg of protein) was added to 2.4 ml of a medium containing 80mM-K hydroxyethyl-piperazine-ethanesulphonate, pH 6.8 and 1μM-FCCP. After 2 min. at 30°, 2μg of antimycin followed by a varying amount of tris base (0-50μl of 0.5M) was added. The rate of reduction of NAD(P) was followed spectrophotometrically at 340-373mμ on addition of 2.5mM-L-glutamate. In the experiments with intact mitochondria labelled (□-□) the proceedure was the same to the point of addition of antimycin, but in this case 2mM-oxoglutarate and 1mM-phosphate were added, resulting in reduction of NAD(P); tris base was added at this point and the rate of oxidation of $NAD(P)H_2$ was followed on addition of 2mM-NH_4Cl. In both sets of experiments the pH was recorded at the end of the experiment. In the experiments with sonicated mitochondria (○-○) the rate of reduction of NAD was followed on addition of 2.5mM-glutamate to 2.5 ml of a medium containing 10μl of sonicated mitochondria (70μg of protein), 80mM-KCl, 5mM-K HEPES, varying amounts of tris base to produce the pH values shown, 2μg of antimycin and 4mM-NAD.

erythro- and threo-hydroxyaspartate were competitive inhibitors of intramitochondrial reduction of NAD(P) by glutamate (Table 1). After sonication of the mitochondrial suspension only erythro-hydroxyaspartate caused significant inhibition of glutamate dehydrogenase activity.

Aspartate transport. It was observed that with mitochondria which had been preincubated with an uncoupling agent, or with ADP together with phosphate, to remove endogenous substrate, that aspartate was unable to react with aspartate aminotransferase. In Fig. 2 experiments are shown in which rat liver mitochondria had been incubated with 1μM-trifluoromethoxycarbonylcyanide-phenylhydrazone (FCCP) to remove partially endogenous substrate. The suspension was then treated with rotenone to inhibit reoxidation of any NAD(P) subsequently reduced. The addition of oxoglutarate together with malonate (to activate oxoglutarate entry [see Chappell et al., 1967]) caused reduction of mitochondrial NAD(P). The addition of aspartate caused only a small oxidation of $NAD(P)H_2$. Had aspartate been able to penetrate freely, an extensive and rapid oxidation would have occurred since the aspartate aminotransferase, which is very active in mitochondria,

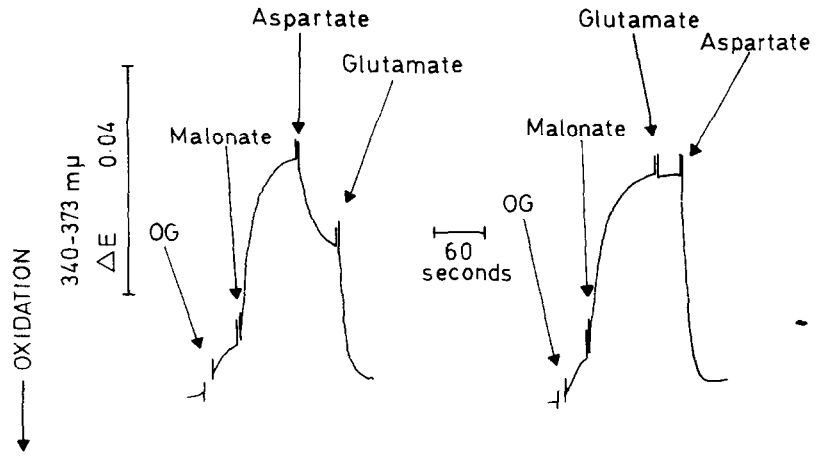

Fig. 2. The activating effect of glutamate on the oxidation of intramitochondrial NAD(P)H_2 by aspartate. Rat liver mitochondria (8 mg of protein) were added to 2.4 ml of a medium containing 1mM-phosphate, 80mM-KCl and 20mM-tris chloride, pH 7.4. After 2 min, 2μg of antimycin followed by 1mM-oxoglutarate were added. The extent of reduction was small (the recorder was reset to allow for the absorbancy artefact due to oxoglutarate) until 1mM-malonate was added to activate oxoglutarate entry. Then 1mM-aspartate followed by 1mM-glutamate, or glutamate followed by aspartate were added as shown. Temperature 30°.

would catalyze the production of oxaloacetate from oxoglutarate and aspartate, and the malate dehydrogenase, which is also very active, would have caused rapid oxidation of NAD(P)H_2, using the oxaloacetate so produced. But on addition of glutamate, which is of course itself a reductant, a rapid and extensive oxidation did occur. The activating effect was also observed when glutamate was replaced by aminoadipate, hydroxyglutamate or hydroxy-aspartate.

The technique of following mitochondrial swelling in iso-osmotic solutions of the ammonium salts of glutamate and aspartate (see Chappell & Haarhoff, 1967) revealed that glutamate penetrated readily mitochondria, i.e. the mitochondria became extensively swollen. However with aspartate no or little swelling occurred and the addition of glutamate did not activate swelling. The only swelling that did occur in ammonium aspartate solutions was that which would have been observed on addition of that amount of glutamate to mitochondria suspended in ammonium chloride, and Cl^- is a non-penetrant. An explanation of these findings is that aspartate enters (or leaves) mitochondria on an exchange-diffusion carrier which has a strict one-for-one stoichiometry, whereas glutamate enters by itself on a separate carrier. Thus swelling occurs in glutamate solutions; with

aspartate, since an equal amount of glutamate has to leave the mitochondria for a given amount of aspartate entering, no swelling occurs other than that due to the glutamate itself.

Table 1. The effects of analogues of glutamate on the rate of reduction of NAD(P) by glutamate with intact and sonicated mitochondria.

The experimental design was as described in the legend to Fig. 1. The analogues were present at 5mM.

	INHIBITION (%)			
	0.5mM-GLUTAMATE		2mM-GLUTAMATE	
	Intact	Sonicated	Intact	Sonicated
L-3-hydroxyglutamate	58	7	43	0
L-2-aminoadipate	27	0	9	0
DL-threo-hydroxyaspartate	33	12	20	0
DL-erythro-hydroxyaspartate	52	23	39	18

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