GLUTAMATE TRANSPORT AND THE TRANS-MEMBRANE pH GRADIENT IN ISOLATED RAT-LIVER MITOCHONDRIA

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1. Introduction

Two transport systems for glutamate have been described in rat-liver mitochondria [1], a glutamate-H^+ symport (alternatively described as a glutamate-hydroxyl exchange diffusion carrier), which is inhibited by NEM and some other sulfhydryl reagents [2,3], and an NEM-insensitive antiport system catalyzing an electrogenic exchange of glutamate (plus H^+) for aspartate [4–6]. These translocators can be thought to be functionally linked to the two pathways of glutamate oxidation in rat-liver mitochondria (see [7]) the transamination pathway, oxidizing glutamate to aspartate (plus CO_2), and the oxidative deamination of glutamate, which would require the participation of the glutamate-H^+ symport [8].

The characterization of the NEM-sensitive glutamate transport process as an electroneutral, proton-coupled translocation was based mainly on the observation that iso-osmotic solutions of ammonium glutamate bring about swelling of rat-liver mitochondria [1–3]. The equilibrium distribution of glutamate was found to be dependent on the pH gradient across the mitochondrial membrane [9] (contrast, see [10]). An effect of the mitochondrial energy level on the glutamate-H^+ symport was suggested by Meyer et al. [3] (see also [11]) who reported an inhibition of glutamate-induced mitochondrial swelling by uncouplers, and by Harris et al. [12], who observed a relationship between energy-linked potassium movements and glutamate transport.

Abbreviations: NEM, *N*-ethylmaleimide; FCCP, *p*-Trifluoromethoxy (carbonyl-cyanide)-phenylhydrazone

Kinetic studies of glutamate uptake by isolated mitochondria in the presence of respiratory inhibitors [3,8] showed that the symport system was characterized by a pH optimum of 6.5, a relatively high K_m for glutamate (4–5 mM) and a low V_{max} (10–20 nmol/min.mg protein, at pH 6.5 and 25°C), as compared to other mitochondrial transport systems (cf. [3,8,13]). It can be estimated that at pH 7.4 the maximal rates of transport reported are less than 30% of the rate of deamination of glutamate that can be obtained in isolated rat-liver mitochondria when this process is stimulated by, for instance, malonate, vitamin K_3 or an uncoupler [7].

We have reinvestigated the initial rate of uptake of glutamate and H^+ in isolated rat-liver mitochondria by the inhibitor-stop method (see [8]). The results demonstrate that, under appropriate conditions, a cotransport of glutamate ions and protons can be observed with a stoichiometry approaching 1 and an initial rate sufficient to account for the metabolic capacity of the mitochondria. Effects of the mitochondrial energy level are best explained as secondary to the actual glutamate-H^+ cotransport.

2. Materials and methods

Rat-liver mitochondria were prepared in 0.25 M sucrose as described by Myers and Slater [14]. Short-time incubations were carried out at 25°C in thermostated vessels (ϕ 1 cm) with continuous stirring. The standard reaction medium for uptake experiments contained 50 mM Tris–HCl, pH 7.4, 45 mM KCl, 2 mM EDTA, 5 mM $MgCl_2$, 5 μg rotenone and 1 mM glutamate in a final volume of 1 ml. ^3H_2O (0.4 μCi) and

either [^{14}C] glutamate or [^{14}C] sucrose (0.2 μCi) were added. Unless otherwise stated, reactions were started by adding an aliquot of the mitochondrial suspension. When changes in the pH of the medium were to be recorded, the incubation medium contained 3 mM Tris–HCl, 100 mM KCl, 2 mM EDTA and 5 μg rotenone. The final pH varied from 7.05–7.20. Reagents to be added were adjusted to the pH of the medium. After each individual incubation the buffer capacity was checked by addition of a known quantity of KOH and HCl. T pH of the medium was registered with a Beckman pH electrode, connected to a Beckman Expandomatic SS-2 pH meter and recorded on a Sargent-Welch recorder.

The inhibitor mixture used to stop glutamate uptake contained 1 mM bromocresolpurple plus 0.1 mM NEM [8]. In experiments where a simultaneous pH recording was made, the use of bromocresolpurple was avoided and reactions were stopped with 0.8 mM NEM. In our hands, neither of these inhibitors were completely effective; consequently, some accumulation of glutamate occurred even when the inhibitors were present from the start of the incubation (see Results). Exactly 30 s after adding the inhibitor mixture, a 0.4 ml sample of the uncubation mixture was centrifuged for 1 min in a Beckman microfuge, model 152. After removing the supernatant, the walls of the centrifuge tube were carefully dried and the pellet was extracted with $HClO_4$ 10%. After removal of the protein, ^{14}C- and ^{3}H-radioactivity was determined in the extracts and supernatants, using a Packard liquid scintillation counter. The scintillation fluid contained toluene/ethanol (7:3 v/v) plus 6 g 2,5-diphenyloxazole and 60 mg, 1,4-bis(4-methyl-5-phenoxazole-2,4)-benzene/litre. Accumulated glutamate was calculated after correction for the amount of glutamate carried in the sucrose accessible space. The volume of the sucrose-accessible space, determined in parallel incubations, varied from 72–82% of the total pellet volume in the experiments reported. Protein was determined by the Biuret method, using bovine serum albumin as the standard. Radioactive material was obtained from New England Nuclear, Chicago. Valinomycin was kindly donated by Dr D. B. Nelson, Stockholm and nigericin by Dr K. Van Dam, Amsterdam. FCCP was a gift from Dr P. Heitler of DuPont. Other chemicals were obtained from British Drug Houses or Sigma Chemical Company.

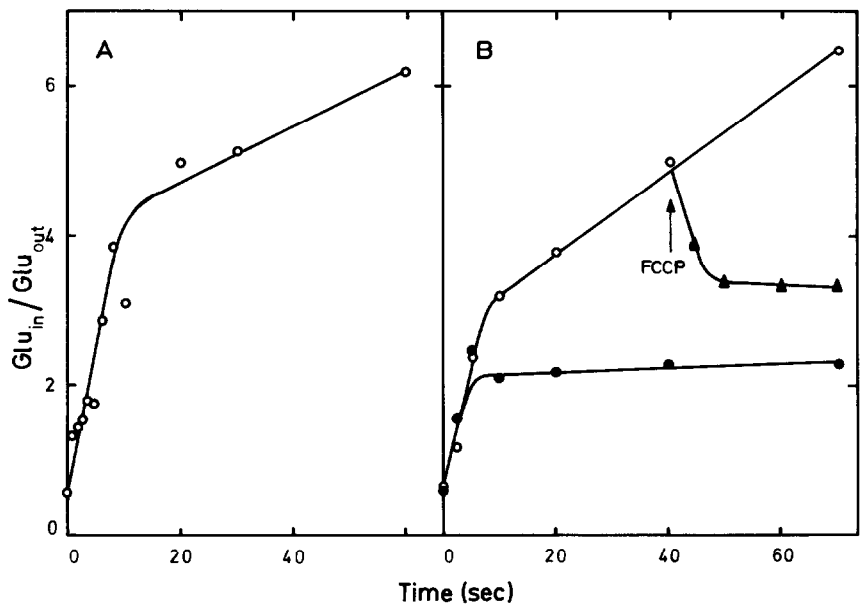

Fig.1. Uptake of glutamate in rat-liver mitochondria and the effect of FCCP. Experimental conditions, see Materials and methods. Glutamate 1 mM, mitochondrial protein 2.6 mg/ml (exp. A) or 2.5 mg/ml (exp. B). (○) control (●) 1 μM FCCP was present from the start of the reaction. (▲) 1 μM FCCP was added after 40 s reaction time.

3. Results

The uptake of glutamate by rat-liver mitochondria followed a strikingly non-linear time course (fig.1A). Under the conditions used here, glutamate was accumulated at a rate of 31 nmol/min.mg protein during the first 5–10 s (in other experiments, the initial rate ranged from 24–35 nmol/min. mg protein), after which the rate of uptake decreased sharply to about 2.4 nmol/min.mg protein (range in other experiments: 2.4–6.0 nmol/min.mg protein). This rate was maintained linearly for at least 70 s.

When the uncoupler FCCP was included in the reaction mixture (fig.1B), the initial rate of uptake of glutamate was unaffected, but the slow, secondary phase of the reaction was almost completely abolished. The addition of FCCP 40 s after starting the reaction caused a release of the accumulated glutamate (cf. [12]).

Concomitant with the rapid uptake of glutamate, protons were removed from the incubation medium. As shown in fig.2, the addition of glutamate to mitochondria equilibrated in a medium of low buffering capacity brought about a rapid increase in pH, the extent of which was dependent on the concentration of glutamate added. A second addition of glutamate caused a similar, but smaller change in pH (results not shown). The glutamate-induced pH change was sensitive to NEM.

Mersalyl, an inhibitor of phosphate transport in rat-liver mitochondria [15] was reported to have little effect on the glutamate translocator [2,3]. As shown in fig.3A, under experimental conditions, mersalyl had a considerable inhibitory effect on the uptake of glutamate when the inhibitor was present from the start of the incubation. Only when mersalyl was added after a 2 min preincubation of the mitochondria, did the subsequent addition of glutamate result in an accumula-

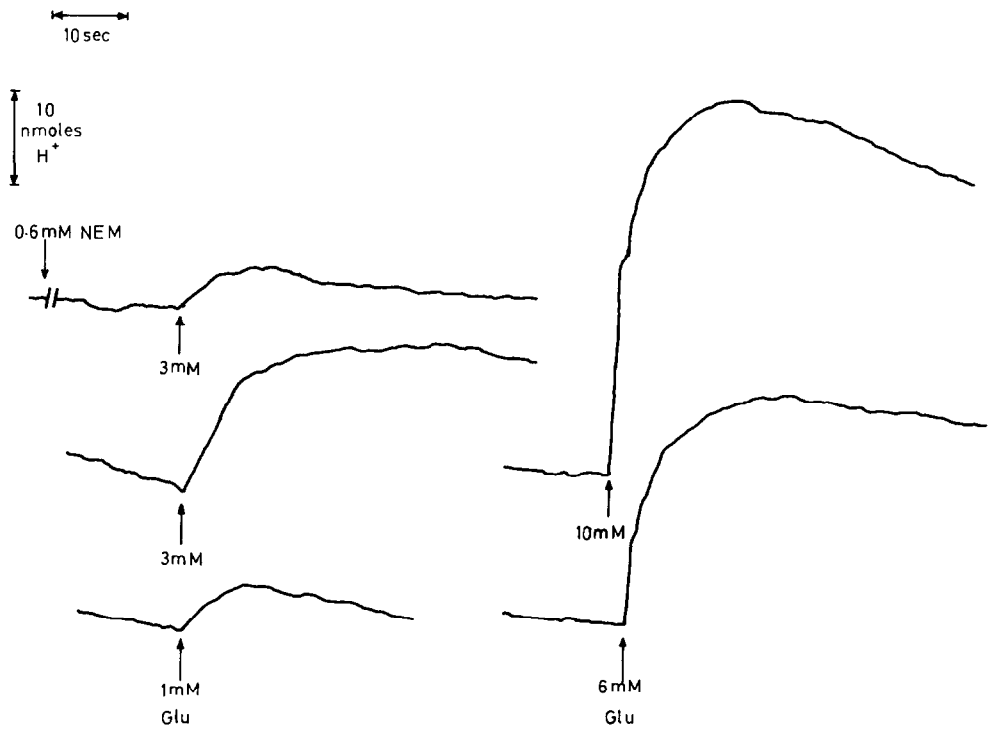

Fig.2. Glutamate-induced pH changes. Mitochondria (1.9 mg/ml) were incubated in a medium (1.6 ml) of low buffer capacity as described in Materials and methods. After equilibration for 3–5 min, additions were made as indicated. The curves are corrected for the small imbalance between the pH of the glutamate stock solution and the pH of the medium. The pH at the time of addition of glutamate varied from 7.12–7.20 in these experiments.

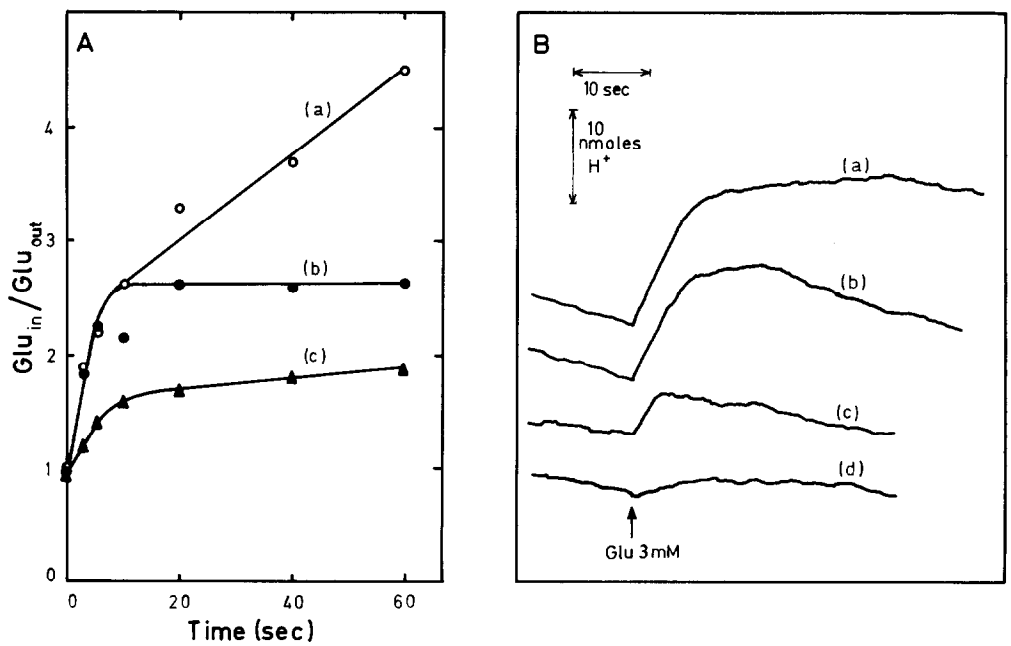

Fig.3. Effect of mersalyl on the uptake of glutamate (A) and on glutamate-induced pH changes (B). Experimental conditions as described in Materials and methods. Mitochondria were preincubated for 2 min. Reactions were started by addition of 1 mM glutamate (exp. A) or 3 mM glutamate (exp. B). (a) control. (b) 0.4 mM mersalyl added after 1 min preincubation. (c) 0.4 mM mersalyl present during the preincubation. Mitochondrial protein 2.1 mg/ml (exp. A) or 1.9 mg/ml (exp. B). In incubation (d) of exp. B the medium contained 1.5 mM K-phosphate instead of Tris-buffer.

tion with the same initial rate as observed in the control. The secondary phase of glutamate uptake was completely inhibited. Similar effects of mersalyl were found on the glutamate-induced pH change (fig.3B). Mersalyl prevented the glutamate-induced pH change only when it was present from the start of the incubation (curve c), but not when added after equilibration of the mitochondria (curve b). These experiments indicate that the initial release of endogenous phosphate is essential for the rapid uptake of glutamate under these conditions (cf. [16]). This interpretation is confirmed by the fact that the addition of glutamate caused no change in pH when the medium contained a low concentration of phosphate (curve d).

The stoichiometry of glutamate and proton uptake was studied in the experiment of table 1. In the standard incubation medium, a ratio of glutamate and proton uptake close to 1 was maintained only during the first five seconds of the reaction; during the secondary phase of the reaction glutamate accumulation significantly exceeded proton uptake. In contrast, when mersalyl was added (after equilibration of the mitochondria) the amount of glutamate accumulated was approximately equal to the amount of H^+ disappearing from the medium throughout the incubation time.

When mitochondria are incubated in a potassium-containing medium in the presence of valinomycin and a source of metabolic energy, a considerable pH gradient across the mitochondrial membrane can be established [17]. Nigericin, which brings about a 1:1 exchange of K^+ and H^+ [18] dissipates an existing pH gradient in a medium containing a high concentration of potassium. The effect of these antibiotics on the uptake of glutamate is shown in fig.4. Nigericin largely prevented the accumulation of glutamate. In contrast, in the presence of valinomycin the rapid phase of glutamate uptake was extended. (A minor leak in the rotenone block would presumably account for the provision of energy required; cf. ref. [2]). These findings further identify the pH gradient across the mitochondrial membrane as a requirement for a rapid uptake of glutamate.

Table 1
Stiochiometry of glutamate-H^+ uptake

Reaction time (seconds)	H^+-uptake (nmoles)	Glutamate uptake (nmoles)	Ratio Glu/H^+
A No mersalyl present			
0	9	10	1.1
$2\frac{1}{2}$	13	18	1.4
5	17	19	1.1
10	13	24	1.8
25	21	29	1.5
50	21	38	1.8
B Mersalyl present			
0	8	9	1.1
3	12	17	1.4
6	12	12	1.0
20	20	18	0.9
40	21	19	0.9

Experimental conditions as described in Materials and methods and in the legend to fig.2. Glutamate, 3 mM; mitochondrial protein, 3.1 mg/ml. At the times indicated, uptake of glutamate was stopped with 0.8 mM NEM. For the calculation of the amount of H^+ taken up, the pH curves were corrected for the drift in pH observed before addition of glutamate. The values for glutamate and H^+ uptake at time zero represent the amounts taken up in an incubation where NEM was added 2 min before the addition of glutamate. Exp. A, No mersalyl added. Exp. B, 0.4 mM mersalyl added 3 min before the addition of glutamate.

4. Discussion

The experimental results reported here demonstrate that glutamate can be rapidly transported across the mitochondrial membrane. This transport is sensitive to NEM and bromocresolpurple and is coupled to the movement of an equivalent amount of protons from the medium when secondary ion movements are prevented. The initial rate of this process is sufficient to account for the rate of deamination of glutamate that can be attained in isolated mitochondria [7]. It seems likely that the glutamate transport studied by others [3,8,12] is equivalent to the secondary, slow phase of uptake observed in our experiments. In the time-course experiments reported both by Harris et al. [12] and

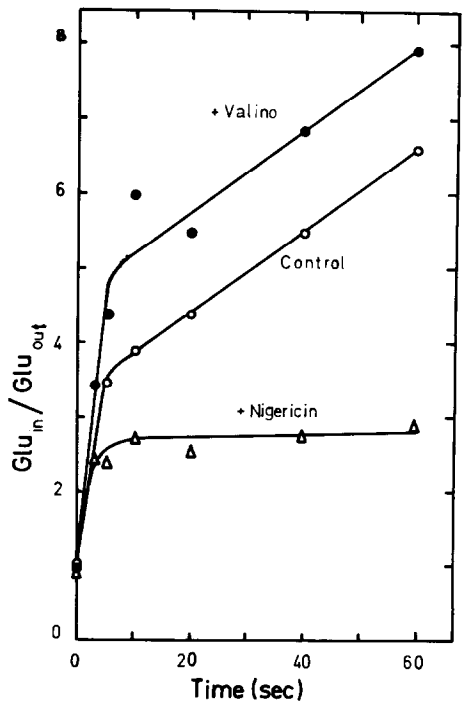

Fig. 4. Effect of valinomycin and nigericin on glutamate uptake. Glutamate, 1 mM; mitochondrial protein 3.8 mg/ml. Valinomycin (1 μg/ml and nigericin (1 μg/ml) were added as ethanolic solutions where indicated.

by Meyer and Vignais [3] the curves do not pass through the origin on extrapolation to the time of addition of glutamate. In the study by Bradford and McGivan [8], mitochondria were preincubated in the presence of an uncoupler before addition of glutamate. This may have led to a dissipation of the conditions required for rapid uptake of glutamate (see below).

The effects of mersalyl on glutamate uptake indicate that an appreciable accumulation can occur only when a pH gradient (alkaline inside) is established, for instance by the release of endogenous phosphate. The effects of valinomycin and nigericin are consistent with this interpretation.

Once the pH difference is neutralised by the protons accompanying the glutamate, further accumulation would be dependent on the capacity of the mitochondria to remove incoming protons, for instance by phosphate reentering the mitochondria in exchange for endogenous anions (citrate, malate; cf. [16]). The inhibition by mersalyl of the secondary phase of glutamate uptake is supporting this interpretation. The overall process would be an exchange of glutamate for endogenous anions. An alternative pathway could be a K^+/H^+ exchange, which could explain the requirement of glutamate uptake for potassium, shown by Harris et al. [12]. Under our conditions this pathway does not appear to contribute significantly to the transport process.

The lack of effect of FCCP on the initial phase of glutamate transport shows that the glutamate translocator per se is not affected by the mitochondrial energy level. On the basis of our experiments, the nature of the inhibition by uncouplers of the slow phase accumulation of glutamate (fig.1B) and of glutamate-induced swelling of mitochondria [2] cannot be established.

The data presented here indicate that the kinetic characteristics of the glutamate-H^+ symport system require re-evaluation. At present, it cannot be excluded that in the studies reported thus far, secondary processes were limiting the rate of uptake of glutamate. This work is now being conducted in our laboratory.

Acknowledgements

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