Glutamate and Aspartate Transport in Rat Brain Mitochondria

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1. Rat brain mitochondria did not swell in iso-osmotic solutions of ammonium or potassium (plus valinomycin) glutamate or aspartate, with or without addition of uncouplers. 2. Glutamate was able to reduce intramitochondrial NAD(P)⁺; aspartate was able to cause partial re-oxidation. 3. These effects were inhibited by *threo*-hydroxyaspartate in whole but not in lysed mitochondria. 4. The existence of a 'malate-aspartate shuttle' for the oxidation of extramitochondrial NADH was demonstrated. This shuttle requires the net exchange of glutamate for aspartate across the mitochondrial membrane. 5. Extramitochondrial glutamate did not inhibit intramitochondrial glutaminase under conditions in which the inhibition in lysed mitochondria was virtually complete. 6. The glutaminase activity of these mitochondria was not energy-dependent. 7. We conclude that these mitochondria do not possess a glutamate-hydroxyl antiporter similar to that of liver mitochondria nor a glutamate-glutamine antiporter similar to that of pig kidney mitochondria, but that they do possess a glutamate-aspartate antiporter.

The transport systems for glutamate and aspartate across the inner mitochondrial membrane have been studied in several tissues. Rat liver mitochondria possess a glutamate-hydroxyl antiporter and a glutamate-aspartate antiporter (Azzi *et al.*, 1967). Pig kidney mitochondria also contain a glutamate-glutamine antiporter (Crompton & Chappell, 1973). Heart mitochondria have only the glutamate-aspartate antiporter, whereas blowfly flight-muscle mitochondria have none of these carriers (Chappell, 1968).

Glutamate and aspartate transport have not previously been reported for brain mitochondria, despite the well established compartmentation of glutamate metabolism in brain tissue (Lajtha *et al.*, 1960; Simon *et al.*, 1967, 1968; Berl *et al.*, 1970*a,b*). It is thought (Van den Berg, 1973) that there is a flow of 4-aminobutyrate, derived from glutamate in inhibitory nerve terminals, across the synapse to post-synaptic and non-synaptic mitochondria where the amino group is passed to 2-oxoglutarate to yield glutamate. It has been postulated (Van den Berg & Garfinkel, 1971; Van den Berg, 1972) that glutamine is formed in the post-synaptic and non-synaptic cells and is returned to the pre-synaptic region. This mechanism is necessary in order to avoid accumulation of amino groups in the post-synaptic and non-synaptic cells at the expense of the pre-synaptic ones.

Glutamate may thus be produced in the matrix of post- and non-synaptic mitochondria by transamination of 4-aminobutyrate with 2-oxoglutarate to yield succinic semialdehyde and glutamate, and utilized in the cytosol of the same cells to form glutamine. For these reactions to occur glutamate may have to cross the mitochondrial membranes. We have

therefore studied the permeability properties of rat brain mitochondria of mainly non-nerve-ending origin to glutamate and aspartate. A preliminary report containing some of this work has been presented (Brand & Chappell, 1973).

Experimental

Rat brain mitochondria were isolated from albino Wistar rats as described by Clark & Nicklas (1970). The washed and diced fore-brains were homogenized by eight or nine passes of a glass-glass Dounce homogenizer with a total clearance of 50–75 μ m.

Protein was assayed by a biuret method (Gornall *et al.*, 1949) incorporating 1.5% (w/v) deoxycholate to solubilize the mitochondria. Other methods are described in the legends to the Figures and Tables.

All biochemicals were of the highest grade commercially available. Enzymes were obtained from Boehringer Corp (London) Ltd., London W.5, U.K. DL-*threo*- β -Hydroxyaspartic acid and DL-*erythro*- β -hydroxyaspartic acid were obtained from Calbiochem, Los Angeles, U.S.A. Amino-oxacetate (carboxymethoxylamine) was from Aldrich Chemical Co., Milwaukee, Wis., U.S.A.

Results

These brain mitochondria exhibit typical mitochondrial swelling in iso-osmotic solutions of permeant solutes such as 4-aminobutyrate or ammonium acetate (Brand & Chappell, 1973, 1974). However, when mitochondria were suspended in 250 mosM-ammonium glutamate or -ammonium

Fig. 1. Swelling of brain mitochondria in iso-osmotic solutions

Mitochondria (1 mg of protein) were suspended in 2.5 ml of the various iso-osmotic solutions (a, b, c and d), each containing $2\mu\text{g}$ of antimycin A/ml and 5 mM-Tris chloride, pH 7.4, at 25°C . ΔE_{640} was measured. (a) 125 mM-ammonium glutamate or 125 mM-ammonium aspartate; (b) 125 mM-KCl; (c) 100 mM-ammonium phosphate; (d) 125 mM-ammonium acetate.

aspartate, no change in light scattering at 640 nm was observed (Fig. 1), showing that neither glutamate nor aspartate was able to enter by exchange with hydroxyl (see Chappell *et al.*, 1972). Similarly no swelling was observed on addition of $10\mu\text{M}$ -carbonyl cyanide phenylhydrazone (an uncoupler), showing that these anions could not enter by electrophoretic uniport. The same lack of swelling was shown in 250 mosm-potassium glutamate or -potassium aspartate in the presence of valinomycin (a potassium ionophore) with or without uncoupler. The mitochondria exhibited rapid swelling in 250 mosm solutions of ammonium phosphate or ammonium acetate, and in potassium phosphate in the presence of valinomycin and uncoupler, showing that they were capable of normal swelling.

Glutamate is, however, metabolized in the mitochondrial matrix, since glutamate is readily oxidized (Brunngraber *et al.*, 1963; Clark & Nicklas, 1970), and we have observed that glutamate is able to stimulate oxidation of malate, presumably by transamination with oxaloacetate in the mitochondrial matrix.

Fig. 2 shows experiments in which penetration was assayed by the ability of extramitochondrial metabolites to reduce intramitochondrial NAD(P)^+ (Chappell, 1968). Glutamate caused a rapid reduction of NAD(P)^+ ; subsequent addition of aspartate

Fig. 2. Reduction and oxidation of intramitochondrial nicotinamide nucleotides by glutamate and aspartate

Rat brain mitochondria (1 mg of protein) were suspended in 0.6 ml of a medium containing 180 mM-mannitol, 75 mM-sucrose, 5 mM-Tris phosphate, 10 mM-Tris chloride, $50\mu\text{M}$ -EDTA, 5 mM-KCl and $10\mu\text{M}$ -carbonyl cyanide phenylhydrazone at pH 7.4, 30°C . After 10–15 min 0.25 mg of antimycin A/ml was added. NAD(P)H formation was followed fluorimetrically. Subsequent additions are marked by arrows and were: 8.3 mM-potassium glutamate, 11 mM-potassium aspartate, 5 mM-potassium oxoglutarate and $100\mu\text{M}$ -potassium malate.

Table 1. *Hydroxyaspartate inhibition of reduction by glutamate and reoxidation by aspartate of mitochondrial nicotinamide nucleotides*

For experimental details see legend to Fig. 2. The experiments with 'broken' mitochondria were similar to those with 'whole' mitochondria except that 0.25mg of NAD⁺/ml and 1mg of Triton X-100 /ml were present throughout. Initial rates of reduction or oxidation were measured.

Conditions	Inhibition of reduction by glutamate (%)		Inhibition of oxidation by aspartate (%)	
	Whole	Broken	Whole	Broken
1. With 8.3 mM-glutamate, 11 mM-aspartate $\pm$ 2.5 mM- <i>threo</i> -hydroxyaspartate	61	<1	62	<1
2. With 16 mM-glutamate, 11 mM-aspartate $\pm$ 2.5 mM- <i>threo</i> -hydroxyaspartate	43	<1	43	<1
3. With 8.3 mM-glutamate, 5 mM-aspartate $\pm$ 2.5 mM- <i>threo</i> -hydroxyaspartate	61	<1	60	<1
4. With 8.3 mM-glutamate, 11 mM-aspartate $\pm$ 2.5 mM- <i>erythro</i> -hydroxyaspartate	53	100	49	—

Table 2. *Oxidation of extramitochondrial NADH by the 'malate-aspartate shuttle'*

Mitochondria (1 mg of protein) were suspended in 2.5 ml of a medium containing 180 mM-mannitol, 75 mM-sucrose, 5 mM-Tris phosphate, 10 mM-Tris chloride, 50 μ M-EDTA and 5 mM-KCl, pH 7.4, at 25°C. NADH oxidation was measured continuously at 340 nm with a Unicam SP. 1800 spectrophotometer. Additions as shown were made at intervals of about 30 s. Final concentrations were: 0.25 mM-NADH, 2.7 mM-potassium aspartate, 4 mM-potassium malate, 2.7 mM-potassium glutamate, 10 μ g of malate dehydrogenase/ml and 20 μ g of aspartate aminotransferase/ml.

Additions	Rate of NADH oxidation (nmol $\cdot$ min ⁻¹ $\cdot$ mg of protein ⁻¹)
1. NADH	12
Aspartate	12
Malate	14
Glutamate	14
Malate dehydrogenase	14
Aspartate aminotransferase	60
2. NADH	15
Aspartate aminotransferase	18
Malate dehydrogenase	18
Malate	20
Aspartate	24
Glutamate	45
3. NADH	12
Malate dehydrogenase	12
Aspartate aminotransferase	12
Glutamate	12
Malate	12
Aspartate	60
4. NADH	20
Aspartate aminotransferase	22
Malate	22
Glutamate	20
Aspartate	40
Malate dehydrogenase	50
5. NADH	20
Malate dehydrogenase	20
Aspartate aminotransferase	22
Glutamate	20
Aspartate	44
Malate	42

caused partial reoxidation of the NAD(P)H, presumably because of oxaloacetate formed by aspartate aminotransferase (EC 2.6.1.1) being used by the malate dehydrogenase (EC 1.1.1.37). A requirement for glutamate was not shown for the reoxidation by aspartate, as similar traces were obtained when glutamate was replaced by 2-oxo-glutarate together with catalytic concentrations of malate to permit entry (Brand & Chappell, 1974). This is presumably because addition of glutamate is unable to increase the total intramitochondrial pool of glutamate together with aspartate, since in this experimental system glutamate may enter only in exchange for aspartate formed from glutamate by transamination. In liver mitochondria glutamate may enter by glutamate-hydroxyl exchange, and then leave in exchange for aspartate, thus stimulating reoxidation of NAD(P)H by extramitochondrial aspartate. In brain mitochondria glutamate-hydroxyl exchange cannot occur, so addition of glutamate does not stimulate reoxidation by extramitochondrial aspartate.

Evidence that a glutamate-aspartate exchange was operating is shown in Table 1. *threo*-Hydroxyaspartate and *erythro*-hydroxyaspartate are known to inhibit glutamate-aspartate exchange in liver mitochondria (Azzi *et al.*, 1967). It can be seen that *threo*-hydroxyaspartate inhibited reduction by glutamate and reoxidation by aspartate in whole but not in broken mitochondria, consistent with inhibition at the site of entry of these anions. *erythro*-Hydroxyaspartate inhibited more in broken mitochondria than in whole; this can be interpreted either as inability to enter together with partial inhibition of the carrier and strong inhibition of the intramitochondrial enzymes, or a partial ability to enter with or without inhibition of the carrier, together with inhibition of the intramitochondrial enzymes.

Further evidence for a glutamate-aspartate exchange is shown in Table 2. A 'shuttle' for the oxidation of extramitochondrial NADH as proposed by Borst (1963) will operate only when malate,

Fig. 3. Glutaminase activity of rat brain mitochondria in the presence and absence of glutamate

Glutaminase was assayed by measuring ammonia formation with an NH_4^+ -sensitive glass electrode with a silver/silver chloride reference electrode in K^+ -free solution. Mitochondria (0.5 mg/mg of protein) were suspended in 5 ml of a medium containing 150 mM-choline chloride, 4 mM-tetramethylammonium phosphate and 10 mM-Tris chloride, pH 7.4, at 30°C . The assay was calibrated for each experiment by the addition of a known amount of NH_4Cl solution. (a) Whole mitochondria; (b) mitochondria which had been frozen and thawed five times; ●, no glutamate present; ○, 2.5 mM-tetramethylammonium glutamate present.

malate dehydrogenase, aspartate, aspartate amino-transferase and glutamate are present in the extra-mitochondrial medium (Chappell, 1969). Table 2 shows that this was true for all constituents except malate and malate dehydrogenase, which may have been present at sufficient activity owing to broken or 'leaky' mitochondria. The Borst shuttle requires the operation of a net glutamate-aspartate exchange, which must therefore have been occurring in this system.

If glutamate enters by exchange with aspartate and not by glutamate-hydroxyl exchange or electrogenic glutamate uptake, then addition of glutamate to a medium containing mitochondria will be unable to raise the intramitochondrial concentration of glutamate plus aspartate, since no glutamate ion will enter until an aspartate ion has been produced in the mitochondrion by transamination and is available for exchange. This was tested by observing the effect of added glutamate on the initial rate of intramitochondrial glutaminase (EC 3.5.1.2) as shown in Fig. 3. Glutamate is a potent inhibitor of both brain glutaminases (see Katanuma *et al.*, 1967); however, in intact mitochondria little significant inhibition was observed, although controls with frozen-thawed mitochondria were completely inhibited by glutamate. Uncoupling by the addition of $10 \mu\text{M}$ -carbonyl cyanide phenylhydrazone had no effect on the rate of ammonia production in whole mitochondria, showing that

the glutaminase activity was not energy-linked as it is in pig kidney mitochondria (Crompton & Chappell, 1973).

Reduction of intramitochondrial NAD(P)^+ by glutamate was almost completely abolished by 1 mM-amino-oxyacetate, a potent aminotransferase inhibitor. This is in agreement with the lack of a glutamate-hydroxyl antiporter, so that entry of glutamate was necessarily followed by transamination (or accompanied by it; see Klingenberg, 1970). Similarly 1 mM-sodium arsenite inhibited reduction by glutamate. Arsenite inhibited re-formation of oxaloacetate which was necessary for this transamination, by inhibiting 2-oxoglutarate dehydrogenase (EC 1.2.4.2).

Discussion

The results of the swelling experiments show that glutamate and aspartate are unable to enter these mitochondria by electrophoretic uniport or by anion-hydroxyl exchange (formally equivalent to anion-proton symport; see Chappell *et al.*, 1972). This conclusion is supported by the lack of inhibition of intramitochondrial glutaminase by extramitochondrial glutamate in whole mitochondria, but not in broken mitochondria. Glutamate is, however, oxidized rapidly and stimulates oxidation of malate. It also shows other effects on the matrix such as

reduction of intramitochondrial NAD(P)^+ , and must therefore be able to enter by exchange with another anion, or by symport with a cation, or to be able to pass reducing equivalents to the matrix by some other mechanism. The inhibition of reduction by glutamate and oxidation by aspartate of intramitochondrial nicotinamide nucleotides by *threo*-hydroxyaspartate and the reconstitution of the Borst shuttle favour the interpretation that a glutamate-aspartate antiport similar to that observed for rat liver mitochondria (Azzi *et al.*, 1967) can occur in rat brain mitochondria.

Miller *et al.* (1973) have estimated that the NADH/NAD^+ ratio in brain mitochondria is 500 times higher than in the cytosol of brain. A similar phenomenon has been observed in liver and in heart. Chappell (1969) has pointed out that in liver this is due to the difference in activities of the component metabolites of the Borst shuttle across the mitochondrial membrane. This could occur by energy input at either the glutamate-aspartate antiporter or the 2-oxoglutarate-malate antiporter or both. We have previously shown the presence of a 2-oxoglutarate-malate antiport in brain mitochondria (Brand & Chappell, 1973, 1974) and the results presented in this paper show that a glutamate-aspartate antiporter is present and that the malate-aspartate shuttle for the oxidation of extramitochondrial NADH can operate in brain mitochondria *in vitro*. It is therefore likely that brain mitochondria maintain a high NADH/NAD^+ ratio in the same way as do liver mitochondria, i.e. by maintaining a difference in the activities of the anions involved across the mitochondrial membrane, probably at the stage of glutamate-aspartate antiport.

Glutamine is oxidized by these mitochondria, and intramitochondrial glutaminase (Salganicoff & De Robertis, 1965) is able to produce NH_3 from extramitochondrial glutamine. Since uncouplers have no effect on the rate of glutaminase as measured by NH_3 production, a glutamate-glutamine antiporter of the type proposed for pig kidney mitochondria (Crompton & Chappell, 1973) is not present in these rat brain mitochondria.

It follows from these conclusions that the only way net translocation of the amino group of glutamate across the mitochondrial membrane can occur is by net permeation of a precursor or product of glutamate. In this way 4-aminobutyrate causes net entry of amino groups into the mitochondria when it enters (Brand & Chappell, 1974) and reacts with 2-oxoglutarate to form glutamate and succinic semialdehyde. If no mechanism exists for the removal of these amino groups from the mitochondria and into glutamine for return to the pre-synaptic regions (Van den Berg & Garfinkel, 1971; Van den Berg, 1972), the latter will rapidly become depleted of

amino nitrogen during nervous activity and thus be unable to function effectively.

It is possible that net export of glutamate from matrix to cytosol could occur by an 'alanine-pyruvate shuttle'. Alanine aminotransferase (EC 2.6.1.2) occurs in both the mitochondrial matrix and the cytosol in brain (Salganicoff & De Robertis, 1965). Thus the following reactions could occur in order to cause net glutamate translocation (subscripts 'in' and 'out' refer to mitochondrial matrix and cytosol respectively):

This scheme requires that 2-oxoglutarate and alanine leave the mitochondria and pyruvate enters. We have shown that these mitochondria are permeable to 2-oxoglutarate (Brand & Chappell, 1973, 1974), alanine (Halling *et al.*, 1973) and pyruvate (A. P. Halestrap, M. D. Brand & R. M. Denton, unpublished work).

The glutamate-hydroxyl antiporter of liver causes accumulation of glutamate within the matrix. Since the opposite effect is required in these brain mitochondria it may not be unreasonable for glutamate efflux to occur via an alanine-pyruvate shuttle, which would tend if anything to extrude glutamate.

There are two major pools of glutamate metabolism in brain (see Berl *et al.*, 1970b). Cheng & Nakamura (1972) have divided the smaller of these two pools into two subdivisions on the basis of the analysis of experiments with labelled glutamate. The smaller glutamate pool probably corresponds to post- and non-synaptic cells; we suggest that the apparent inhomogeneity of this pool may be due to the inability of glutamate or aspartate to penetrate the mitochondrial membrane except by strict antiport or by way of metabolism through, for example, an alanine-pyruvate shuttle.

Katanuma *et al.*, (1967) have suggested that glutaminase in brain is not functioning owing to the high concentrations of glutamate present. The absence of a glutamate-hydroxyl antiporter means that the concentration of glutamate in the micro-environment of glutaminase may be very much less than in whole tissue. Thus glutaminase may well be able to function actively in brain tissue.

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