Intramitochondrial Localization of the 4-Aminobutyrate–2-Oxoglutarate Transaminase from Ox Brain

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In order to determine the intramitochondrial location of 4-aminobutyrate transaminase, mitochondria were prepared from ox brain and freed from myelin and synaptosomes by using conventional density-gradient-centrifugation techniques, and the purity was checked electron-microscopically. Inner and outer membranes and matrix were prepared from the mitochondria by large-amplitude swelling and subsequent density-gradient centrifugation. The fractions were characterized by using both electron microscopy and different marker enzymes. From the specific activity of the 4-aminobutyrate transaminase in the submitochondrial fractions it was concluded that this enzyme is associated with the inner mitochondrial membrane.

It is generally agreed that the 4-aminobutyrate–2-oxoglutarate transaminase (EC 2.6.1.19) from brain is mainly associated with free mitochondria (Salganicoff & De Robertis, 1963, 1965; van den Berg *et al.*, 1965; van Kempen *et al.*, 1965; Balázs *et al.*, 1966; Waksman *et al.*, 1968; Reijnierse *et al.*, 1975), and a preparation of a crude mitochondrial fraction was used by Schousboe *et al.* (1973) and Maitre *et al.* (1975) as a powerful purification step in the preparation of a homogeneous 4-aminobutyrate transaminase from mouse or rat brain. No direct evidence seems, however, to be available about the intramitochondrial localization of this enzyme, but on the basis of experiments in which mitochondria were treated with Triton X-100, Salganicoff & De Robertis (1965) suggested that it should be a matrix enzyme.

Knowledge about the precise intramitochondrial localization of the 4-aminobutyrate transaminase is of importance not only for the understanding of the function of the enzyme (cf. Schousboe *et al.*, 1974) but also for the interpretation of results related to the compartmentation of glutamate and 4-aminobutyrate metabolism in brain (van den Berg & Garfinkel, 1971; Reijnierse *et al.*, 1975). In order to obtain such information, inner- and outer-membrane fractions were prepared from purified mitochondria and characterized by using marker enzymes as well as electron microscopy.

Materials and Methods

Chemicals

Malate dehydrogenase, NAD⁺, NADH, NADP⁺ and cytochrome *c* were purchased from Boehringer, Mannheim, Germany. Heparin (sodium salt) and

pyridoxal phosphate were from Sigma Chemical Co., St. Louis, MO, U.S.A. Ficoll was from Pharmacia, Uppsala, Sweden, and crystallized bovine serum albumin was from BDH Biochemicals, Poole, Dorset, U.K. 4-Amino[1-¹⁴C]butyrate (sp. radioactivity 50 mCi/mmol) and [1-¹⁴C]tyramine (sp. radioactivity 9 mCi/mmol) were obtained from New England Nuclear Corp., Boston, MA, U.S.A. All other chemicals were of the purest grade available from regular commercial sources.

Preparation of mitochondria and membrane fractions

Ox brains obtained from the slaughterhouse after removal from the skull were placed in ice-cold 0.4M-sucrose containing 20 mM-Hepes,‡ pH 7.2, 2 mM-EDTA and 0.02% (w/v) heparin (medium A). After mincing, the tissue was homogenized at 4°C in 5 vol. of medium A by using a Potter–Elvehjem homogenizer with a motor-driven Teflon pestle (Corona Mess-technik, Lorch, W. Germany). After re-adjustment of the pH to 7.2 with 2M-KOH, medium A was added to the homogenate to give a total volume of 15 ml of medium A/g fresh wt. of brain. The mixture was centrifuged for 20 min at 4°C and 1000g and the supernatant was re-centrifuged for 15 min at 4°C and 12000g. The pellet was resuspended in 4 ml of 10% (w/v) Ficoll in medium A/g fresh wt. and centrifuged for 30 min at 4°C and 12000g. The supernatant was re-centrifuged for 30 min at 4°C and 35000g and the pellet resuspended in medium A (0.5 ml/g fresh wt.). This procedure for the preparation of a crude mitochondrial fraction is a slight modification of the method of Basford (1967). The mitochondria were freed from synaptosomes and

‡ Abbreviation: Hepes, 2-(N-2-hydroxyethyl)piperazin-N'-yl)sulphonic acid.

myelin by the method of Gray & Whittaker (1962), except that a Ficoll gradient was used instead of a sucrose gradient. The suspension of the crude mitochondria was layered on top of a discontinuous gradient consisting of 20% (w/v) Ficoll in medium A and 10% (w/v) Ficoll in medium A and centrifuged for 120 min at 4°C and 75000g in a Beckman ultracentrifuge equipped with a SW 27.1 rotor. The purified mitochondria obtained as the pellet after this centrifugation were used for the preparation of inner and outer membranes as described by Marks (1974) and for electron microscopy (see below). The purified mitochondria were suspended (2–3 mg of protein/ml) in a hypo-osmotic medium consisting of 20 mM-potassium phosphate, pH 7.2, 0.02% (w/v) bovine serum albumin and 0.02% (w/v) heparin (medium B) and kept at 4°C for 20 h. Then it was layered on top of 10% (w/v) Ficoll in medium A and centrifuged for 30 min at 4°C and 30000g in a Beckman ultracentrifuge (SW 60 rotor), giving three fractions: the clear supernatant on top of the 10% (w/v) Ficoll was taken as the mitochondrial matrix, the layer on top of the 10% Ficoll as outer mitochondrial membranes and the pellet as inner mitochondrial membranes. Inner and outer membranes were resuspended in small volumes of medium B (4–6 mg of protein/ml).

Mitochondria from rat liver were prepared as described by Johnson & Lardy (1967). The large-amplitude swelling of these mitochondria in hypo-osmotic medium was performed as described above for the brain mitochondria. The swollen mitochondria were centrifuged for 60 min at 4°C and 100000g in a Beckman ultracentrifuge equipped with a SW 60 rotor. The supernatant was taken as the matrix fraction and the pellet, which represents the mitochondrial-membrane fraction, was resuspended in a small volume of medium B (6–8 mg of protein/ml).

Electron microscopy

Suspensions of purified brain mitochondria and inner and outer membranes were centrifuged at high speed (30 min at 4°C and 100000g) and the resulting pellets fixed in 3% (v/v) glutaraldehyde (16 h) and post-fixed in 1% (w/v) OsO₄ (2 h). Both solutions were buffered with 0.1 M-sodium cacodylate, pH 6.8. The pellets were then stained in 0.5% (w/v) uranyl acetate and embedded in Araldite.

The blocks were cut on an LKB ultramicrotome and the sections post-stained in 2% (w/v) uranyl acetate and Reynold's lead citrate. The stained sections were examined in a Philips 201 C electron microscope.

Enzyme assays

Cytochrome *c* oxidase (EC 1.9.3.1) activity was measured at 27°C as described by Smith (1955) and

fumarase (EC 4.2.1.2) activity (27°C) by the method of Racker (1950). Rotenone-insensitive NADH-cytochrome *c* reductase (EC 1.6.99.3) activity was assayed at 27°C by the method of Sottocasa *et al.* (1967). NADP⁺-dependent isocitrate dehydrogenase (EC 1.1.1.42) activity was determined at 37°C as described by Bernt & Bergmeyer (1974). Aspartate-2-oxoglutarate transaminase (EC 2.6.1.1) activity was determined at 37°C by the enzymic assay of Bergmeyer & Bernt (1974), by using 0.1 M-Tris/HCl buffer, pH 8.0, containing 20 mM-L-aspartate, 4 mM-2-oxoglutarate, 0.02 mM-pyridoxal phosphate, 0.1 mM-NADH and 10 units of malate dehydrogenase (EC 1.1.1.37)/ml. Monoamine oxidase (EC 1.4.3.4) activity was measured at 37°C as described by McCaman *et al.* (1965) by using 2.5 mM-[¹⁴C]-tyramine (sp. radioactivity 40–50 d.p.s./nmol) as the substrate. 4-Aminobutyrate transaminase activity was determined at 37°C by using a modification of the assay described by Hall & Kravitz (1967). The assay mixture consisted of 0.1 M-Tris/HCl, pH 8.0, 1 mM-aminoethylisothiuronium bromide, 0.02 mM-pyridoxal phosphate, 4 mM-2-oxoglutarate, 1 mM-succinate, 3 mM-NAD⁺ and 25 mM-4-amino[¹⁴C]-butyrate (sp. radioactivity 5–8 d.p.s./nmol). Blanks were run in the same buffer minus 2-oxoglutarate. The ¹⁴C-labelled reaction products (succinic semi-aldehyde and succinate) were separated from 4-amino-[¹⁴C]butyrate on Dowex 50 (X2; 50–100 mesh) columns (0.7 cm × 7 cm). Specific enzyme activities are expressed as units/mg of protein, where 1 unit is defined as that activity catalysing the conversion of 1 μmol of substrate/min at the temperature of the assay. Radioactivity was determined as described by Schousboe & Hertz (1971) in a Packard Tri-Carb liquid-scintillation spectrometer. Protein was measured by the method of Lowry *et al.* (1951) as modified by Miller (1959), by using bovine albumin as the standard. Statistical analyses were performed by using Student's *t* test.

Results

Electron microscopy

Plate 1 shows an electron micrograph of the purified brain mitochondria. They appear relatively homogeneous, with only a few synaptosome-like bodies, and have an intact double-membrane structure. The subfractions obtained after large-amplitude swelling are shown in Plates 2 and 3, and this treatment has disrupted the mitochondria, which appear as 'ghosts' or empty vesicles. It has, however, not been adequate for a complete separation of the inner and outer membrane. Fully detached inner membranes are only seen in some instances (Plate 2), and the outer-membrane fraction (Plate 3) is contaminated with inner membranes still in contact with outer membranes.

EXPLANATION OF PLATE I

Electron micrograph of mitochondria (M) purified from ox brain

The mitochondria appear intact, with double-membrane structures. A few synaptosome-like bodies (S) are seen.

EXPLANATION OF PLATE 2

Electron micrograph of the mitochondrial inner-membrane fraction

Most of the vesicles are identifiable as morphologically damaged mitochondria. In some cases outer membranes are seen (arrow).

EXPLANATION OF PLATE 3

Electron micrograph of the mitochondrial outer-membrane fraction

Empty vesicles of different sizes surrounded by a single membrane predominate. In some of the vesicles segments with double-membrane structures are seen.

Table 1. Specific activities of 4-aminobutyrate transaminase and six marker enzymes in ox brain mitochondria and inner membranes, outer membranes and matrix prepared from the mitochondria by large-amplitude swelling

Specific activities are expressed as units/mg of protein. For details of enzyme assays, see the Materials and Methods section. The protein contents of the different fractions were corrected for the serum albumin added to the swelling medium. Results are averages $\pm$ S.E.M. of four or five individual experiments.

Fraction	4-Aminobutyrate transaminase		Aspartate transaminase		Isocitrate dehydrogenase		Fumarase		Cytochrome <i>c</i> oxidase		Rotenone-insensitive NADH-cytochrome <i>c</i> reductase		Monoamine oxidase		Protein	
	(munits/mg)	(%)	(units/mg)	(%)	(munits/mg)	(%)	(units/mg)	(%)	(units/mg)	(%)	(munits/mg)	(%)	(munits/mg)	(%)	(%)	(%)
Mitochondria	15.9 $\pm$ 1.2	100	1.31 $\pm$ 0.15	100	28.0 $\pm$ 4.1	100	0.58 $\pm$ 0.11	100	1.51 $\pm$ 0.16	100	14.2 $\pm$ 0.7	100	9.2 $\pm$ 0.9	100	100	100
Inner membrane	21.5 $\pm$ 1.2	60	2.11 $\pm$ 0.18	71	41.0 $\pm$ 5.1	41	0.64 $\pm$ 0.11	49	2.02 $\pm$ 0.22	59	21.2 $\pm$ 1.5	66	11.0 $\pm$ 0.9	53	44	44
Outer membrane	12.7 $\pm$ 1.6	14	1.80 $\pm$ 0.27	24	16.3 $\pm$ 2.3	26	0.59 $\pm$ 0.10	17	1.72 $\pm$ 0.12	20	29.0 $\pm$ 4.2	35	12.1 $\pm$ 2.0	23	17	17
Matrix	8.3 $\pm$ 2.2	8	1.29 $\pm$ 0.46	15	22.8 $\pm$ 4.0	25	0.10 $\pm$ 0.05	3	0.03 $\pm$ 0.01	0	6.0 $\pm$ 2.2	6	2.8 $\pm$ 0.9	5	15	15
Recovery (%)		82		110		92		69		79		107		81		76

Biochemical findings

The specific activities of the 4-aminobutyrate transaminase and the marker enzymes in the brain mitochondrial fractions are shown in Table 1. Comparison of activities in the submitochondrial fractions with those of the intact mitochondria shows that there is a statistically significant ($P < 0.01$) increase in the specific activity in the inner-membrane fraction for 4-aminobutyrate transaminase and aspartate transaminase, whereas the increases in specific activities observed for isocitrate dehydrogenase and cytochrome *c* oxidase are on the borderline of significance ($0.10 > P > 0.05$). In the outer-membrane fraction the rotenone-insensitive NADH-cytochrome *c* reductase had a significantly higher activity ($P < 0.01$), whereas the increase in specific activity for monoamine oxidase was statistically not significant ($P > 0.2$). All enzymes except aspartate transaminase and isocitrate dehydrogenase showed significantly ($P < 0.01$) decreased activities in the matrix fraction.

The activities of isocitrate dehydrogenase and aspartate transaminase in the subfractions of the liver mitochondria are shown in Table 2. The specific activity of the isocitrate dehydrogenase is significantly higher ($P < 0.025$) in matrix than in the intact mitochondria, whereas the specific activity in the membrane fraction is significantly lower ($P < 0.05$). The transaminase had, however, a slightly higher specific activity in the membrane fraction, whereas its specific activity was lower in matrix than in the mitochondria.

Discussion

As judged from the electron micrographs (Plates 1-3) the brain mitochondria used in the present study were highly purified, which is a prerequisite when a mitochondrial localization of a brain enzyme is to be studied (cf. Marks, 1974). The large-amplitude swelling used for the preparation of membrane fractions is not the most efficient one for either liver (Parsons *et al.*, 1966) or brain mitochondria (D'Monte *et al.*, 1970), but it has the advantage that no detergents that might interfere with enzymes are added. It did, however, lead to an almost complete disruption of the mitochondria, ensuring that true matrix enzymes would be liberated and subsequently found in the supernatant after centrifugation.

The increase in specific activity of cytochrome *c* oxidase in inner membranes and of rotenone-insensitive NADH-cytochrome *c* reductase and monoamine oxidase in outer membranes is in agreement with that found for liver, heart and brain mitochondria (Schnaitman *et al.*, 1967; Craven *et al.*, 1969; Kropp & Wilson, 1970; Watanabe, 1971;

Table 2. *Specific activities of isocitrate dehydrogenase and aspartate transaminase in liver mitochondria and membranes and matrix prepared from the mitochondria by large-amplitude swelling*

Specific activities are expressed as units/mg of protein. For details of enzyme assays, see the Materials and Methods section. The protein contents of the different fractions were corrected for the serum albumin added to the swelling medium. Results are averages $\pm$ S.E.M. of three or four individual experiments.

Fraction	Isocitrate dehydrogenase		Aspartate transaminase		Protein (%)
	(munits/mg)	(%)	(munits/mg)	(%)	
Mitochondria	57.3 $\pm$ 9.7	100	60.7 $\pm$ 15.2	100	100
Membranes	22.0 $\pm$ 7.7	20	73.3 $\pm$ 12.1	71	57
Matrix	114.8 $\pm$ 16.5	80	37.7 $\pm$ 8.0	25	39
Recovery (%)		100		96	96

Hayashi & Capaldi, 1972; Addink *et al.*, 1972). Even though about 25% of the isocitrate dehydrogenase activity was recovered in the matrix fraction, this enzyme as well as the aspartate transaminase and fumarase were found to be associated with the inner membrane in brain mitochondria, which is not in accord with results obtained from liver mitochondria, in which these enzymes have been found in the matrix (Schnaitman & Greenawalt, 1968; Brdiczka *et al.*, 1968). It should, however, be emphasized that these enzymes have been reported to be membrane-bound in heart mitochondria (Smoly *et al.*, 1968; Wit-Peters *et al.*, 1971; Addink *et al.*, 1972). That great differences may exist between mitochondria from different organs is further supported by the results obtained with swollen liver mitochondria (Table 2), since the large-amplitude swelling solubilized about 80% of the isocitrate dehydrogenase activity from these. That most of the aspartate transaminase also in the liver mitochondria was associated with the membrane fraction after the swelling may indicate that this enzyme, even in liver mitochondria, may be solubilized only after mechanical disruption or treatment with detergents.

Like cytochrome *c* oxidase, the 4-aminobutyrate transaminase had the highest specific activity in the inner membranes, and 60% of the enzyme activity was recovered in this fraction, whereas only 8% was found in the matrix. It is therefore concluded that this enzyme is associated with the inner mitochondrial membrane. This disagrees with Salganicoff & De Robertis (1965), who concluded that it was localized in the matrix on the basis of experiments in which Triton X-100 was used to disrupt the mitochondria. Such a treatment might well have solubilized the 4-aminobutyrate transaminase, since in the present study it was observed that a 30s sonication of either a brain homogenate or the purified mitochondria liberated between 80 and 90% of the 4-aminobutyrate transaminase activity into a high-speed supernatant (results not shown). It has also been shown by Wu *et al.* (1976) that about 80% of the 4-aminobutyrate transaminase activity in brain could

be solubilized by two consecutive homogenizations in water. All this evidence indicates that the transaminase is relatively loosely bound to the membrane.

The observation that the 4-aminobutyrate transaminase is a membrane-bound enzyme should be kept in mind when the kinetic properties of the enzyme are discussed. The properties of highly purified enzyme preparations (Schousboe *et al.*, 1973; Maitre *et al.*, 1975; John & Fowler, 1976) may well be different from the properties of the native enzyme, which is surrounded by a more or less apolar environment in the mitochondrial membrane. It may accordingly be difficult to correlate measurements of fluxes through the 4-aminobutyrate shunt (Balázs *et al.*, 1970; Machiyama *et al.*, 1970) in intact tissue preparations with activities of the 4-aminobutyrate transaminase determined in disintegrated preparations. This is indicated by the discrepancy between the calculated rates of the transaminase reaction in mouse brain (van den Berg & Garfinkel, 1971) and the activity determined in a water homogenate (Wu *et al.*, 1976).

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