Selective Permeability of Rat Liver Mitochondria to Purified Aspartate Aminotransferases *in vitro*

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1. A method was devised to allow determination of intramitochondrial aspartate aminotransferase activity in suspensions of intact mitochondria. 2. Addition of purified rat liver mitochondrial aspartate aminotransferase to suspensions of rat liver mitochondria caused an apparent increase in the intramitochondrial enzyme activity. No increase was observed when the mitochondria were preincubated with the purified cytoplasmic isoenzyme. 3. These results suggest that mitochondrial aspartate aminotransferase, but not the cytoplasmic isoenzyme, is able to pass from solution into the matrix of intact rat liver mitochondria *in vitro*. 4. This system may provide a model for studies of the little-understood processes by which cytoplasmically synthesized components are incorporated into mitochondria *in vivo*.

Studies of the biogenesis of mitochondria have so far been restricted almost entirely to attempts to understand the processes of protein biosynthesis within the organelle and to identify the translation products of this system; these studies have now made considerable progress (for reviews, see Ashwell & Work, 1970; Schatz & Mason, 1974). It is evident, on the other hand, that the majority of mitochondrial proteins are coded by the nuclear genome and synthesized on cytoplasmic ribosomes. Knowledge of the processes of incorporation of cytoplasmic translation products into intact or developing mitochondria is, however, sparse. Some progress has been made. For example, Godinot & Lardy (1973) showed that, after administration of radioactive isoleucine to rats, labelled liver glutamate dehydrogenase appeared first associated with microsomal fractions and was subsequently transferred to the mitochondria. Similarly, Kadenbach (1967) has demonstrated transfer of cytochrome *c* from microsomal particles to mitochondria *in vitro*. Systems such as these appear, however, to have eluded further analysis.

We decided to investigate further the processes by which cytoplasmically synthesized proteins are incorporated into mitochondria and have chosen aspartate aminotransferase (EC 2.6.1.1) as the specific system for study. This enzyme is of particular interest since it exists in two molecular forms in the

cells of most higher organisms (Boyd, 1961; Wada & Morino, 1964), one associated with the soluble fraction and the other with the mitochondria; Baumber & Doonan (1976) have shown that the localization of the two forms is unique in that no mitochondrial isoenzyme is detectable in the cytoplasmic fraction from rat liver and vice versa. The two isoenzymes from any particular organism differ widely in chemical, physical, catalytic and immunochemical properties (Wada & Morino, 1964), but it has been shown (Doonan *et al.*, 1974) that the structures of the isoenzymes from pig heart muscle are homologous. Little is known about the biosynthesis of the mitochondrial isoenzyme, but studies with somatic cell hybrids (van Heyningen *et al.*, 1974) have shown that it is coded by the nuclear genome and is therefore probably synthesized on cytoplasmic ribosomes.

In this case therefore there exists not only the problem of how a cytoplasmically synthesized protein becomes incorporated into the mitochondria, but also that of how the incorporation procedure selects between two structurally related isoenzymes; as pointed out above, the process of selection is very highly specific. In the present paper, evidence is presented to show that purified rat liver mitochondrial aspartate aminotransferase can pass from solution into the matrix space of intact rat liver mitochondria in the absence of any other cytoplasmic components, whereas entry of the homologous cytoplasmic isoenzyme does not occur. This would appear to provide a very useful system for further studies of the assembly of functional mitochondria.

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Materials and Methods

Materials

Male white Wistar rats weighing approx. 250 g were used throughout. L-Aspartic acid and 2-oxoglutaric acid were from BDH Chemicals, Poole, Dorset, U.K. Cycloserine and amino-oxyacetic acid were from Sigma Chemical Co., St. Louis, MO, USA. Malate dehydrogenase (10 mg/ml of glycerol) was from Boehringer, Mannheim, West Germany. CM-cellulose CM-52 was from Whatman Biochemicals, Springfield Mill, Maidstone, Kent, U.K. Rotenone was from F.P. Penick and Co., New York, NY, USA. 2-Oxo[^{14}C]glutaric acid (sodium salt) was from The Radiochemical Centre, Amersham, Bucks., U.K.

Preparation of suspensions of rat liver mitochondria

Rat liver mitochondria were prepared as previously described (Klingenberg & Slenczka, 1959). After final centrifugation, the mitochondrial pellet was suspended in a solution containing 250 mM-sucrose, 20 mM-Tris/HCl buffer, pH 7.4, and 1 mM-EGTA to a final protein concentration of 50–60 mg/ml; protein concentrations were determined by the method of Waddell (1956). Mitochondrial suspensions were kept over ice and used within 2 h of preparation.

Assays of solubilized aspartate aminotransferase

These were carried out by the method of Karmen (1955) with the modified substrate concentrations of Baumber & Doonan (1976); with these substrate concentrations, the measured activities of both isoenzymes were maximal.

Purification of cytoplasmic and mitochondrial aspartate aminotransferases from rat liver

The purification procedures were based on those described for the isoenzymes from pig heart (Banks *et al.*, 1968; Barra *et al.*, 1976).

Cytoplasmic enzyme. An homogenate in 250 mM-sucrose was prepared from two livers (approx. 14 g of tissue) and the nuclei and mitochondria were removed by centrifugation at 8000g for 10 min. Protein was precipitated from the resulting supernatant by the addition of $(\text{NH}_4)_2\text{SO}_4$ (0.51 g/ml); the pH was maintained at 7.4 by the addition of NaOH and the temperature at 4°C. Precipitated protein was collected by centrifugation at 30000g for 45 min and the pellet suspended in 0.02 M-sodium acetate buffer, pH 5.4. After dialysis against the same buffer (5 × 3 litres) for a total of 5 h at 4°C, insoluble protein was removed by centrifugation at 10000g for 20 min and the supernatant passed through a column of Sephadex G-25 equilibrated with the same acetate buffer to effect final removal of $(\text{NH}_4)_2\text{SO}_4$; the bed volume of the column was approx. 4 times that of the sample. The protein solution after passage through Sephadex was applied to a column (1.7 cm × 15 cm) of CM-cellulose

CM-52 equilibrated in the same acetate buffer; all the enzyme activity was absorbed to the resin. The enzyme was eluted by application of an acetate buffer of higher concentration (0.08 M) at the same pH; the enzyme emerged with the breakthrough of the new buffer. The protein solution was de-salted and concentrated by an Amicon pressure dialysis system (60 ml cell with a PM-10 membrane).

Mitochondrial enzyme. A suspension of mitochondria (20 mg of protein/ml, 15 ml) prepared from four rat livers as described above was treated with sodium deoxycholate at a final concentration of 1.3% (w/v). Membrane fragments were removed by centrifugation at 105000g for 1 h, after which the supernatant was passed through a column (1.7 cm × 40 cm) of Sephadex G-25 equilibrated with 0.01 M-Tris/HCl buffer, pH 7.8. The resulting protein solution was applied to a column (1.7 cm × 15 cm) of CM-cellulose CM-52 equilibrated with the same Tris/HCl buffer; the enzyme was absorbed on the resin. Elution was effected by application of 0.01 M-Tris/HCl buffer, pH 7.8, containing 0.1 M-NaCl. The enzyme emerged with the breakthrough of the new buffer and was concentrated as described for the cytoplasmic isoenzyme.

Measurement of aspartate aminotransferase activity in intact mitochondria

The measurements were carried out by a fluorescence method. An Eppendorf photometer (model 1101 M) was used in the fluorescence mode equipped with the appropriate filters for measurement of the fluorescence of NAD(P)H. Mitochondria (approx. 3 mg of protein) were incubated at 22°C in a solution of 250 mM-sucrose, 20 mM-Tris/HCl buffer, pH 7.4, and 1 mM-EGTA containing 2 µg of rotenone and 2 mM-sodium arsenite; the total volume was 1 ml. After 3 min, aspartate was added (usually to a final concentration of 9.6 mM) and then after a further 1 min, 2-oxoglutarate was added to a final concentration of 3 mM; stock solutions of both substrates were adjusted to pH 7.4 by the addition of NaOH. The rate of decrease of fluorescence was then recorded and was taken as a measure of the amount of intramitochondrial aspartate aminotransferase. Variations in the procedure that were used to study the effect of other added reagents will be described below.

Results and Discussion

Purification of cytoplasmic and mitochondrial aspartate aminotransferases

Solutions of these two enzymes were obtained with protein concentrations [measured from the A_{280} assuming $A_{280}^{1\%} = 14.0$ (Barra *et al.*, 1976)] of 1.5 and 0.8 mg/ml for the mitochondrial and cytoplasmic isoenzymes respectively. The specific activities were 183 and 291 µ mol of product/min per mg of protein respectively. The isolation of these enzymes has not

been reported previously, but the specific activities obtained were comparable with those of the completely purified mitochondrial and cytoplasmic enzymes from pig heart [Barra *et al.* (1976) and Banks *et al.* (1968) respectively], and from rat brain (Magee & Phillips, 1971). Starch-gel electrophoresis followed by staining for enzyme activity (Baumber & Doonan, 1976) showed that the mitochondrial enzyme preparation was completely free of the cytoplasmic form and vice versa. It was not possible, however, with the limited amount of material available to demonstrate conclusively that the preparations were not contaminated with small amounts of inactive proteins.

Measurement of aspartate aminotransferase activity in intact mitochondria

An outline of the principles of the method is as follows. Mitochondria are preincubated with rotenone and sodium arsenite, which block the operation of the electron-transport chain and of the 2-oxoglutarate dehydrogenase complex respectively, thus allowing reduced nicotinamide nucleotides to accumulate. The mitochondria are then loaded with aspartate after which 2-oxoglutarate is added. Transport of the latter substance into the mitochondrial matrix then provides the complete substrate pair for aspartate aminotransferase and reaction products, glutamate and oxaloacetate, are formed.

Fig. 1. *Effect of external mitochondrial aspartate aminotransferase on the rate of change of fluorescence*

The test systems contained 3.0mg of mitochondrial protein. Aspartate (9.6mM) and 2-oxoglutarate (3mM) were added at the points indicated. (a) No externally added enzyme; (b) enzyme (10 μ g) added at the point indicated. (a) —, Progress curve of the reaction; —, tangent to the initial part of progress curve. In subsequent Figures, only the tangents are shown. The numbers assigned to the tangents are their slopes in arbitrary units of scale divisions/min.

Table 1. *Variation in rate of change of fluorescence with external aspartate concentration*

The test systems contained 1.9mg of mitochondrial protein and the external 2-oxoglutarate concentration was 3mM.

Concentration of external aspartate (mM)	Rate of change of fluorescence (scale divisions/min)
0	0
2.4	25
4.8	29
7.2	35
9.6	42
19.2	40

The oxaloacetate reacts stoichiometrically with reduced nicotinamide nucleotides, the reaction being catalysed by endogenous malate dehydrogenase; the 'malic' enzyme may also contribute to the process. Hence measurement of the rate of decrease of fluorescence of NAD(P)H provides a measure of the rate of intramitochondrial transamination, provided that the malate dehydrogenase reaction is not rate-limiting. That this is not the case is shown by the observation that assay of aspartate aminotransferase in suspensions of disrupted mitochondria by the method of Karmen (1955) does not require the addition of exogenous malate dehydrogenase.

A typical progress curve for decrease in NAD(P)H fluorescence is shown in Fig. 1(a). The initial small decrease in fluorescence on the addition of aspartate was probably due to reaction with endogenous 2-oxoglutarate. After the addition of external 2-oxoglutarate, a rapid essentially linear decrease in fluorescence with time was observed after which the rate decreased until the NAD(P)H was exhausted. The rate of change of fluorescence, and hence the rate of transamination, was taken from the slope of the tangent to the initial part of the curve and expressed in arbitrary units of scale divisions/min. In all other Figures given here, only the tangents to the initial parts of the curves are shown. In duplicate assays a reproducibility of $\pm 10\%$ or better was found.

The substrate concentrations used in standard assays require comment. The optimum concentration of aspartate was determined in a set of experiments (Table 1). The rate of change of fluorescence had reached a maximum at an external aspartate concentration of 9.6mM and this concentration was used in subsequent work. No change in fluorescence occurred in the absence of added aspartate. With a fixed concentration of aspartate (9.6mM), the rate of change of fluorescence was independent of the external concentration of 2-oxoglutarate over the range 0.33–5.5mM; this is consistent with the reported K_m value of approx. 50 μ M for the 2-oxoglutarate carrier

of rat liver mitochondria (Palmieri *et al.*, 1972). A 2-oxoglutarate concentration of 3 mM was used as a routine in the experiments described below.

The validity of the assay procedure clearly requires that the rate of entry of 2-oxoglutarate be greater than the rate of intramitochondrial transamination; slow entry of aspartate is not a problem since adequate time for its accumulation may be allowed before addition of the second substrate. Some support for the view that the assay method measures aspartate aminotransferase activity has been obtained from the effect of the permeant competitive aminotransferase inhibitor amino-oxyacetic acid (Rognstad & Katz, 1970; Meijer & van Dam, 1974). When this inhibitor was added to the assay system to a concentration of 0.6 mM 1 min before the addition of aspartate, a decrease of 30% in the rate of change of fluorescence was observed. Failure to increase the degree of inhibition at higher concentrations of amino-oxyacetate may reflect a limited capacity for this substance in mitochondria. In view of the uncertainty in interpretation of this result, however, two other experimental approaches have been used to validate the assay method.

Attempts were made to measure the rate of uptake of 2-oxo[^{14}C]glutarate by mitochondrial suspensions under the above assay conditions; the 'inhibitor-stop' method was used (Palmieri *et al.*, 1972). Mitochondria (2.5 mg) were incubated in the usual medium (1 ml) for 3 min after which 9.6 mM-aspartate was added. After a further 1 min had elapsed, 3 mM-2-oxo[^{14}C]glutarate was added followed 10 s later by 40 mM-2-phenylsuccinate. The mitochondria were immediately collected by centrifugation for 1 min at full speed in an Eppendorf bench centrifuge (model 3200), washed and the accumulated radioactivity was determined (Palmieri *et al.*, 1971). In control experiments, the order of addition of 2-phenylsuccinate and 2-oxoglutarate was reversed. It was found that in both test and control samples of mitochondria the amount of internal 2-oxoglutarate had reached a maximum in the time taken (approx. 1 min) for incubation and collection of the mitochondria. The amount of radioactivity in the mitochondria was not increased by extension of the time of incubation to 1 min or by preincubation of the mitochondria with purified aspartate aminotransferases. These results show that, at the high concentrations of 2-oxoglutarate used here, the competitive inhibitor of transport, 2-phenylsuccinate, is ineffective in blocking the 2-oxoglutarate entry and that even in its presence, accumulation of oxo acid is complete in less than 1 min. This should be compared with the time-course of decrease in NAD(P)H fluorescence which typically extended over 2–3 min.

In a second type of experiment, the rate of change of fluorescence was measured with a suspension of

mitochondria in the normal way and the rate compared with that obtained from a sample of the same mitochondria after sonication; all conditions were identical except that the sonicated sample was assayed in the presence of added 1 mM-NADH (Table 2). It had previously been shown that sonication exposes all the mitochondrial aspartate aminotransferase activity (Boyd, 1961). Hence the identity of rates with intact and sonicated mitochondria is a clear demonstration that the fluorescence assay method measures aspartate aminotransferase activity and not the rate of 2-oxoglutarate entry.

Experiments were also carried out to assess the extent to which NH_4^+ ions in the incubation medium would interfere with the assay procedure by allowing operation of intramitochondrial glutamate dehydrogenase. The results are given in Table 3. Rates of change of fluorescence were measured under the normal assay conditions, but with various amounts of NH_4Cl added immediately before addition of

Table 2. *Comparison of the rate of change of fluorescence with intact and sonicated mitochondria*

Mitochondria (44 mg of protein/ml) were either suspended in medium A (0.25 M-sucrose/20 mM-Tris/HCl buffer/1 mM-EGTA, pH 7.4) or sonicated (50 W, 0.2 V, 15 s) in medium B (medium A + 1 mM-NADH). Samples (50 μl) were assayed in duplicate in the appropriate medium under the usual conditions.

Mitochondria	Assay medium	Rate of change of fluorescence (scale divisions/min)
Intact	A	42, 42
Sonicated	B	44, 41

Table 3. *Effect of added NH_4^+ on the rate of change of fluorescence*

The test systems contained 3.2 mg of mitochondrial protein. Assays were carried out in the usual way except that various amounts of NH_4Cl were added to the suspensions immediately before addition of aspartate.

Concentration of external NH_4Cl (mM)	Rate of change of fluorescence (scale divisions/min)
0	40, 44
2	40
5	41
10	51
20	60
40	45

aspartate. At low concentrations of external NH_4^+ ion (up to 5 mM) no effect was observed, whereas at 20 mM an increase in rate of change of fluorescence of approx. 50% was apparent. Higher (40 mM) concentrations appeared to be inhibitory. The NH_4^+ ion contents of the samples of purified cytoplasmic and mitochondrial aspartate aminotransferases used in the experiments described below were determined by measurement of the oxidation of NADH in the presence of 2-oxoglutarate and glutamate dehydrogenase. The sample of the mitochondrial isoenzyme was completely free of NH_4^+ ions; the sample of the cytoplasmic form contained trace amounts of NH_4^+ ions, but not sufficient to interfere with the fluorescence assays in experiments where the purified isoenzyme was added to the incubation medium (see below).

Effect of externally added mitochondrial aspartate aminotransferase

The effect of externally added mitochondrial aspartate aminotransferase on the intramitochondrial activity was studied by adding the enzyme to the mitochondrial suspension after the 3 min incubation period with rotenone and sodium arsenite, and after a further 1 min aspartate was added in the usual way. A typical example is shown in Fig. 1(b) with the appropriate control in Fig. 1(a); an increase of 50% in the measured activity is apparent. Fig. 2 shows the results of a more extensive series of experiments where the percentage increase in rate of change of fluorescence over that of the control is plotted against amount of added external enzyme. The increase

Fig. 2. Variation of rate of change of fluorescence with externally added mitochondrial aspartate aminotransferase. The conditions were as described in Fig. 1. Various quantities of enzyme were added 1 min before the addition of aspartate.

reached a maximum of 65% at approx. 5 μg of added enzyme/mg of mitochondrial protein. These results are taken to show that mitochondrial aspartate aminotransferase is able to pass from solution into the mitochondrial matrix.

Problem of externally generated oxaloacetate

A possible objection to the interpretation placed on the observations described above is that oxaloacetate is generated by aspartate aminotransferase from substrates remaining in the suspension medium; entry of this product into the mitochondria could then give rise to the observed increase in rate of change of fluorescence. Transport of oxaloacetate by the 2-oxoglutarate carrier is unlikely to occur due to the low affinity of the former substance for this carrier (Palmieri *et al.*, 1972) and to the high concentration of 2-oxoglutarate used. Entry by the dicarboxylate carrier (Gimpel *et al.*, 1973) could, however, present a problem. Elimination of this source of artifacts was achieved by using the aspartate aminotransferase inhibitor cycloserine (Meijer & van Dam, 1974) as shown in Fig. 3.

When 10 mM-cycloserine was added to the mitochondrial suspension 10 min before the addition of aspartate, no decrease in rate of change of fluorescence was observed compared with a control (Fig. 3a and b); this is consistent with the fact that cycloserine cannot penetrate the mitochondrial inner membrane (Meijer & van Dam, 1974). Addition of mitochondrial aspartate aminotransferase to the mitochondrial suspension (Fig. 3c) gave rise to the usual increase (44%) in rate of change of fluorescence and this increase was completely unaffected by the addition of cycloserine 1 min after addition of the enzyme (Fig. 3d). In a control experiment, purified enzyme and cycloserine were incubated for 10 min at concentrations similar to those pertaining in the experiment of Fig. 3(d); subsequent assay showed complete inhibition of enzyme activity. Hence oxaloacetate could not have been generated externally in the experiment of Fig. 3(d) and it seems to be necessary to conclude that the increase in rate of change of fluorescence was due to an increased amount of intramitochondrial aspartate aminotransferase. Since these rates were identical in Figs. 3(c) and 3(d), it is clear that oxaloacetate, when generated externally, does not interfere with the assay procedure.

Effect of externally added cytoplasmic aspartate aminotransferase

A set of experiments was performed to examine the effect of the addition of the cytoplasmic rather than the mitochondrial isoenzyme of aspartate aminotransferase to mitochondrial suspensions. Some of the results are shown in Fig. 4, from which it can be seen that the addition of 9.6 μg of cytoplasmic isoenzyme did not increase the rate of change of fluorescence;

Fig. 3. Effect of cycloserine on the rate of change of fluorescence

The test systems contained 3.25 mg of mitochondrial protein. Other conditions were as described in Fig. 1. (a) No cycloserine or external enzyme; (b) cycloserine (10 mM) added 10 min before aspartate; (c) enzyme (12 μ g) added 1 min before aspartate (d) enzyme (12 μ g) added 1 min before cycloserine, then aspartate after a further 10 min.

Fig. 4. Effect of external cytoplasmic aspartate aminotransferase on the rate of change of fluorescence

The test systems contained 3.0 mg of mitochondrial protein. Other conditions were as described in Fig. 1. (a) No externally added enzyme; (b) cytoplasmic aspartate aminotransferase (9.6 μ g) added 1 min before aspartate.

the same was true when 16.0 μ g of enzyme was used. In a control experiment with the mitochondrial isoenzyme (10 μ g) an increase of 50% in the rate of change of fluorescence was obtained.

There are two main implications to be drawn from these results. First, since the rates of generation of external oxaloacetate would have been approximately the same in the presence of mitochondrial isoenzyme or of cytoplasmic isoenzyme, the different

effects in the two cases conclusively rules out any possibility of artificial increases in rate due to entry of oxaloacetate into the mitochondria. Secondly, the results suggest that the permeability of mitochondria to aspartate aminotransferase *in vitro* is selective, as might indeed be expected from the unique distributions of the two isoenzymes *in vivo* (Baumber & Doonan, 1976).

Conclusions

Arguments are presented above to support the view that the assay procedure used provides a measure of intramitochondrial aspartate aminotransferase activity. This being so, then the results obtained on incubation of mitochondria with aspartate aminotransferase isoenzymes show clearly that the mitochondrial, but not the cytoplasmic isoenzyme, is able to enter the mitochondria. A way may therefore be available to study the processes by which cytoplasmically synthesized proteins are incorporated into mitochondria. In subsequent studies it may prove to be more convenient to use radioactively labelled isoenzymes to follow the incorporation processes, but the less-direct method used here had the advantage of demonstrating that the added mitochondrial isoenzyme was transported to the site of action of the intramitochondrial isoenzyme [that is, to the matrix (Matlib & O'Brien, 1975)] rather than to some other possible site.

From the results given here, no hypothesis can be formulated about the mechanism of the penetration of the mitochondrial membranes by aspartate aminotransferase; this must await further studies. It should, however, be pointed out that the mitochondrial pre-

paration used could have been contaminated with cytoplasmic ribosomes (Gaitskhoki *et al.*, 1974; Kellems *et al.*, 1975) and hence it is possible that these contribute to the overall process. The probable involvement of ribosomes in transfer of proteins to mitochondria is already apparent from the work of Kadenbach (1967) and of Godinot & Lardy (1973).

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