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Intramitochondrial localization of alanine aminotransferase in rat-liver mitochondria: comparison with glutaminase and aspartate aminotransferase

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Summary. The removal of the outer mitochondrial membrane and hence of constituents of the intermembrane space in rat-liver mitochondria using digitonin showed that phosphate-dependent glutaminase, alanine and aspartate aminotransferase were localized in the mitoplasts. Further fractionation of mitoplasts following their sonication resulted in 90% of glutaminase, 98% of alanine aminotransferase and 48% of aspartate aminotransferase being recovered in the soluble fraction while the remainder of each enzyme was recovered in the sonicated vesicles fraction. These results indicated that glutaminase and alanine aminotransferase were soluble matrix enzymes, the little of each enzyme recovered in the sonicated vesicles fraction being probably due to entrapment in the vesicles. Aspartate aminotransferase had dual localization, in the inner membrane and matrix with the high specific activity in sonicated vesicles confirming its association with the membrane. Activation experiments suggested that the membrane-bound enzyme was localized on the inner side of the inner mitochondrial membrane.

Keywords: Amino acids – Alanine aminotransferase – Aspartate aminotransferase – Glutaminase – Mitochondria

Introduction

There appears to be a sidedness in the deamination by transamination of glutamate in isolated mitochondria, depending on whether glutamate is exogenous or endogenous with more aspartate and alanine being synthesized when glutamate is exogenous (Kovačević, 1971; Masola et al., 1985). The lower rate of aspartate and alanine synthesis with endogenous glutamate, formed from glutamine, did not appear to be due to substrate availability since large quantities of glutamate were produced in rat enterocyte mitochondria (Masola et al., 1985). These findings suggest a different

intramitochondrial localization of alanine and aspartate aminotransferases (EC 2.6.1.2 and EC 2.6.1.1 respectively) and of phosphate – dependent glutaminase (EC 3.5.1.2).

Alanine aminotransferase is known to have cytosolic and mitochondrial isozymes (DeRosa and Swick, 1975). The rat-liver mitochondrial enzyme is however very unstable (Swick et al., 1965). The porcine-liver mitochondrial enzyme which is more stable, was found predominantly in the soluble fraction representing the intermembrane space and matrix in subfractionated mitochondria (DeRosa and Swick, 1975). Aspartate aminotransferase is also known to have cytosolic and mitochondrial isozymes in mammalian tissues (Boyd, 1961; Fleisher et al., 1960). There are, however conflicting reports on the localization of the mitochondrial enzyme with some describing its localization in the matrix (Landriscina et al., 1970; Marco et al., 1969). Other investigators have found the enzyme to be partially bound to the inner mitochondrial membrane on the innerside (Elduque et al., 1982) or outer-side (Gil et al., 1987). Its reversible movement between the inner and outer sides of the inner membrane has also been described (Waksman and Rendon, 1974). Phosphate-dependent glutaminase (PDG) has been shown to be localized in the matrix or inner surface of liver and rat-kidney mitochondria (Shapiro et al., 1985; McGivan et al., 1980) or the outer surface of the inner membrane in pig-kidney mitochondrial (Kvamme et al., 1991).

In this paper we examine the intra mitochondrial localization of alanine aminotransferase in relation to glutaminase and aspartate aminotransferase in sub-mitochondrial fractions.

Material and methods

Male albino rats Wistar strain weighing 200–250 g were used in the present experiments. Biochemicals were obtained from the Sigma Chemical Co, USA. Reagents were of analytical grade.

Isolation and fractionation of mitochondria

Rat-liver mitochondria were prepared by the method of Chappell and Hansford (1972) in an isolation medium containing 250 mM sucrose, 5 mM Hepes and 1 mM EGTA, pH 7.4.

The digitonin fractionation of mitochondria was as described by Schnaitman and Greenawalt (1968) with some modifications. Digitonin treated aliquots of mitochondrial suspensions were not continuously stirred but were thoroughly mixed initially and again at the end of the 20 minute incubations at 0°C. The "low-speed pellet" consisting of mitoplasts and/or intact mitochondria was obtained by centrifuging the digitonin treated mitochondrial suspensions at 25,000 g for 15 minutes. The "high speed pellet and supernatant" were obtained from the supernatant resulting from the low speed centrifugation by centrifuging it at 100,000 g for 60 minutes.

In experiments where mitoplasts and sonicated vesicles were prepared the outer membrane was removed using a digitonin concentration of 1.2 mg/10 mg protein. Attempts to remove the outer membrane using the swelling procedure as with pig-heart mitochondrial (Maisterrena et al., 1974) resulted in only 36% of the outer membrane being removed as judged by the monoamine oxidase activity retained in the "mitoplasts" fraction. Mitoplasts were collected from digitonin treated mitochondria by centrifugation at 25,000 g for 10 minutes following a 20 minute incubation. The mitoplasts were washed

twice to remove residual outer membrane fragments then sonicated using an Artek Sonic Dismember Model 300 operated at 100 W power. Each sample was sonicated three times for periods of 10 seconds at 0°C, allowing cooling periods in between sonications. Unbroken mitoplasts were removed by centrifugation at 25,000 g for 15 minutes. Sonicated vesicles were collected by centrifugation of the supernatant at 100,000 g for 60 minutes.

Enzyme assays and protein determination

Monoamine oxidase was assayed as described by Tabor et al. (1954) by monitoring the formation of benzaldehyde at 250 nm. Adenylate kinase was assayed as described by Bergmeyer (1974). Cytochrome oxidase activity was determined by the spectrophotometric method of Sattocasa, et al. (1967) after activation with lubrol (Schnaitman and Greenawalt, 1968). β-Hydroxybutyrate dehydrogenase was assayed as described by Gotterer (1967) after activation by brief sonication. The activities of glutamate and malate dehydrogenases were assayed at 25°C as described by Schmidt (1974) and McGivan et al. (1980), respectively, following their activation with 0.1% Triton-X-100. Alanine and aspartate aminotransferase activities were determined as described by Volman-Mitchell and Parsons (1974), the latter being activated with 0.1% Triton-X-100. Glutaminase was assayed as described by Curthoys and Weiss (1974). In experiments where alanine aminotransferase and glutaminase activation is indicated this was by brief sonication. Alanine aminotransferase was assayed immediately because of its instability.

Protein was determined by the Biuret method (Gornall et al., 1949) with bovine serum albumin as the standard.

Results

Effect of digitonin on the release of mitochondrial enzymes

The release of enzymes from rat-liver mitochondria following digitonin treatment (Fig. 1a) was similar to that obtained by Schnaitman and Greenawalt (1968). The activities of adenylate kinase, monoamine oxidase (MAO), malate dehydrogenase and cytochrome oxidase are presented as markers of cellular compartments. At low digitonin concentrations adenylate kinase, localised in the intermembrane space (Brdiczka et al., 1968) was released into the high-speed supernatant (Fig. 1c) which represents the intermembrane space. Next to be released was MAO which indicated the removal of the outer membrane (Schnaitman et al., 1967). At higher concentrations (>1.5 mg digitonin/10 mg protein) most of MAO was recovered in the high-speed supernatant indicating solubilization of the outer-membrane (Fig. 1b and c). At 2 mg digitonin/10 mg protein, 80% or more of the activity of all the other enzymes i.e. cytochrome oxidase, malate dehydrogenase, alanine and aspartate aminotransferase and glutaminase remained in the low-speed pellet which is associated with the inner membrane-bound fraction (mitoplasts).

Effect of sonication on the release of enzymes from the mitoplasts of liver mitochondria

The results in Table 1 show that alanine aminotransferase and glutamate dehydrogenase were most readily released by sonication of mitoplasts.

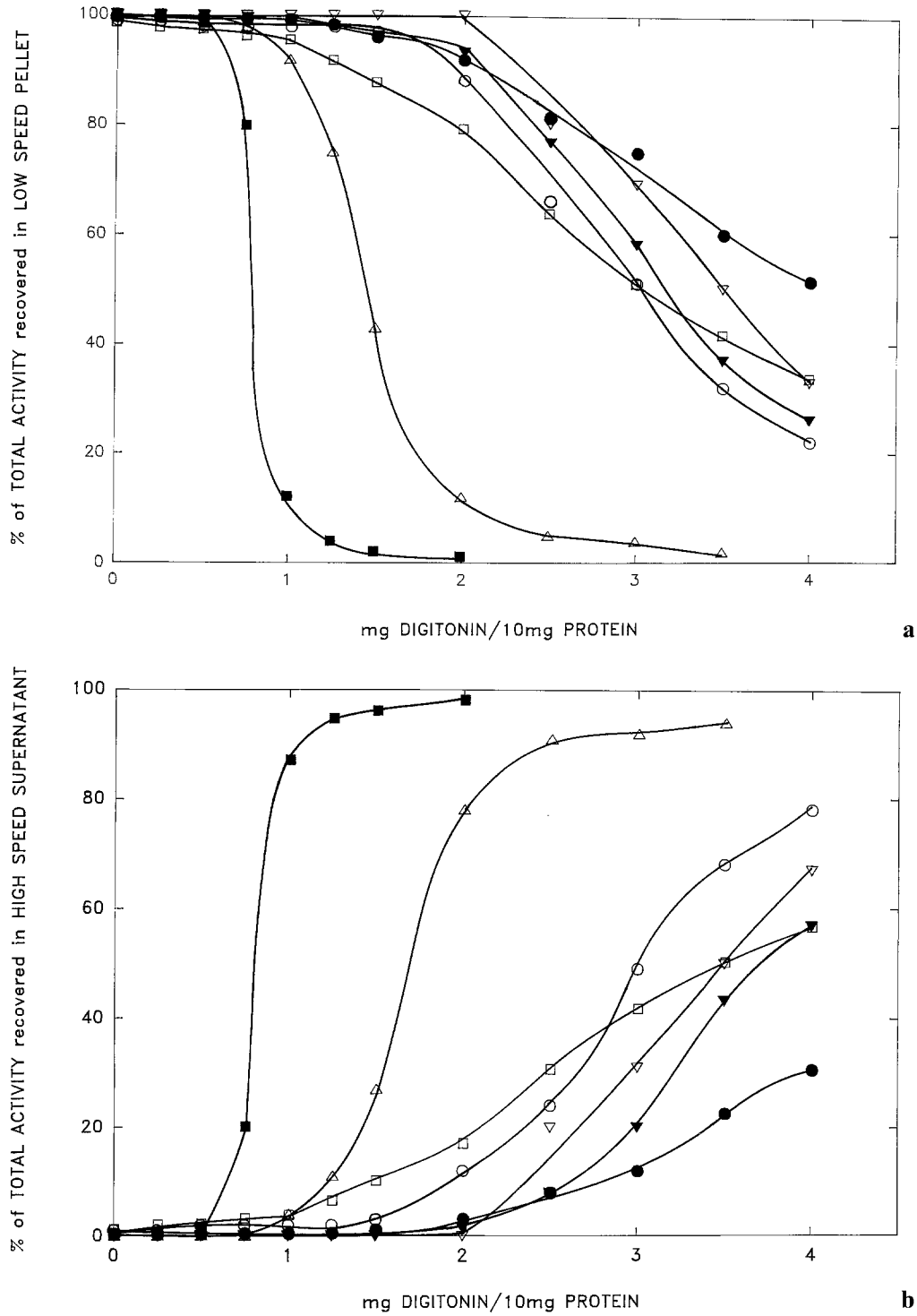

Fig. 1. Recoveries of rat-liver mitochondrial enzymes in (**a**) low speed pellet (**b**) high speed pellet (**c**) high speed supernatant following digitonin treatment of intact mitochondria are expressed as percentages of the total enzyme present in fractions of digitonin treated mitochondria. Each data point represents the average of results from two different mitochondrial preparations. The recovery of enzymes in the digitonin treated fractions expressed a percentage of that in non-treated mitochondria was between 91–105%. Experimental conditions were as described in methods <-■-> adenylate kinase; <-△-> monoamine oxidase; <-□-> malate dehydrogenase; <-▼-> cytochrome oxidase; <-○-> alanine aminotransferase; <-●-> aspartate aminotransferase; <-▽-> glutaminase

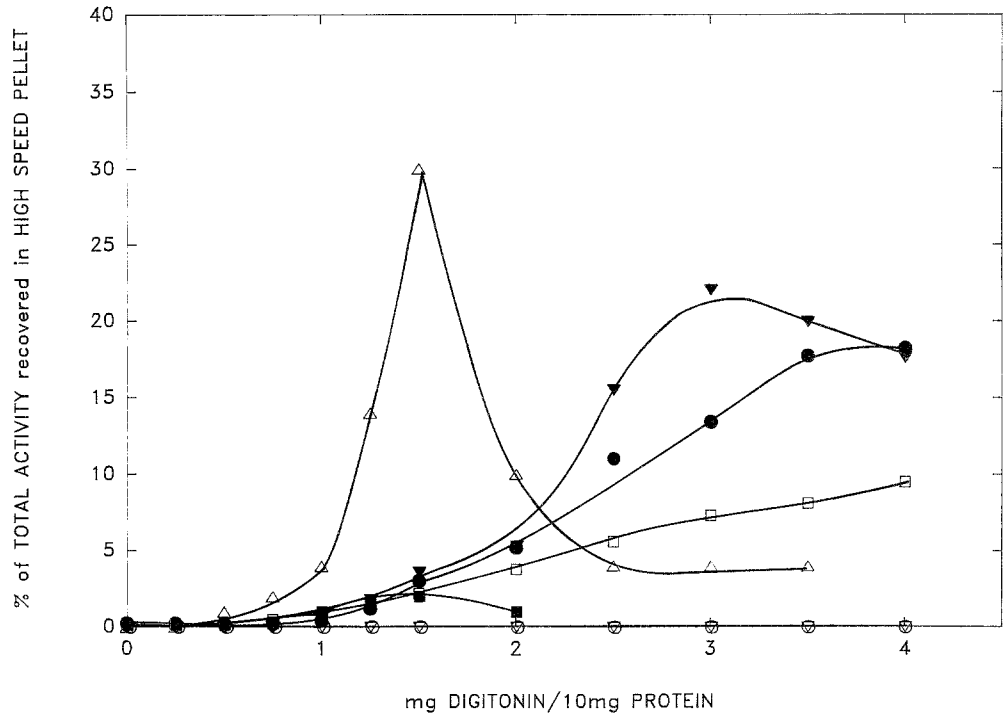

Fig. 1c

Noteworthy was the finding that more that 90% of the enzyme released from mitoplasts was recovered in the supernatant and not in the membranous sonicated vesicles fraction for either enzyme. This points to very little or no association of these enzymes with the inner membrane.

Aspartate aminotransferase, cytochrome oxidase and malate dehydrogenase were less readily released from mitoplasts. Of the enzyme released from mitoplasts, 77% of cytochrome oxidase, 52% of aspartate aminotransferase and 37% of malate dehydrogenase was recovered in the membranous sonicated vesicles indicating association of these enzymes with the inner membrane. If this association was due to entrapment in vesicles then alanine aminotransferase and glutamate dehydrogenase would have been affected. Although the data in Table 1 show that glutaminase was not readily released from mitoplasts, this may be misleading and might only reflect a destruction of the enzyme released during sonication especially in view of the low recovery of 60%.

The specific activities of the enzymes in the sonicated vesicles and supernatant (Table 1) reflected associations of enzymes with either the inner membrane or matrix. Cytochrome oxidase and aspartate aminotransferase had high specific activities in the sonicated vesicles fraction indicating an association with the inner membrane whereas glutamate dehydrogenases, alanine aminotransferase and glutaminase had high specific activities in the supernatant, indicating an association with the matrix.

Table 1. Distribution of enzymes in fractions obtained by the differential centrifugations of mitoplasts following a 3 × 10 seconds sonication

Enzyme	Unbroken mitoplasts	% of Total recovered activity		% Recovery	Enzyme activity in n moles substrate/min/mg protein		
		Sonicated vesicles	Supernatant		Unbroken mitoplasts	Sonicated vesicles	Supernatant
Cytochrome Oxidase	65	27 (77)	8 (23)	99	1,057 ± 133	2,873 ± 141	340 ± 18
Malate dehydrogenase	65	13 (37)	22 (63)	89	2,011 ± 143	1,799 ± 126	1,297 ± 41
Glutamate dehydrogenase	55	3 (7)	42 (93)	99	2,285 ± 160	634 ± 50	3,347 ± 111
Alanine aminotransferase	49	1.0 (2)	50 (98)	97	45.9 ± 4.4	4.3 ± 1.8	91.7 ± 5.2
Aspartate aminotransferase	77	12 (52)	11 (48)	96	1,371 ± 45	1,342 ± 52	481 ± 14
*Glutaminase	80	2.0 (10)	18 (90)	60	38.1	3.9	17.7
Protein	58	13 (31)	29 (69)	98			

The sonicated vesicles and supernatant refer to the pellet and supernatant obtained following the 100,000g, 60 minutes centrifugation of the supernatant resulting from the 25,000g, 15 minutes centrifugation of sonicated mitoplasts. Each result is a mean of four experiments. *, results represent the mean of two experiments. Values of enzyme activity are followed by standard deviation. The figures in parantheses represent the proportion of enzyme expressed as a percentage of the total enzyme released from mitoplasts by sonication. % recovery is total recovered activity expressed as a percentage of activity in unsonicated mitoplasts.

Comparative latency of enzyme activity in mitoplasts and sonicated vesicles

Cytochrome oxidase is located on the outer surface of the inner mitochondrial membrane (Harmon et al., 1974) hence turning mitoplasts inside-out by sonication results in reduced access of the substrate to the enzyme (Maïrouch and Godinot, 1977; Huang et al., 1973). Similarly β-hydroxybutyrate dehydrogenase which is located on the inner surface of the inner mitochondrial membrane is fully exposed in sonicated vesicles compared to mitoplasts (McIntyre et al., 1978). In our experiments (Table 2) the increase in cytochrome oxidase activity due to lubrol activation was 1.7 times for mitoplasts and 2.8 times for sonicated vesicles showing reduced access of the substrate, cytochrome c, in the sonicated vesicles. β-Hydroxybutyrate dehydrogenase activity was increased 1.4 times due to activation by brief sonication and none at all in sonicated vesicles. Further, the specific activity of this enzyme was very high in sonicated vesicles being 4.2 times that in mitoplasts. The above results confirmed the inside-out orientation of the sonicated vesicles.

The specific activity of alanine aminotransferase and glutaminase was very low in sonicated vesicles (Table 2) which was consistent with the low percentages of these enzymes found in this fraction as compared with that in the supernatant fraction (Table 1). Clearly these two enzymes are soluble and located in the matrix since turning mitoplasts inside out resulted in 90% of glutaminase and 98% of alanine aminotransferase activity appearing in the soluble (supernatant) fraction (Table 1). The enzymes did not respond to activation, in fact there was a loss of activity in mitoplasts on sonication. Activation may not be necessary as the substrates, alanine and glutamine are electroneutral at physiological pH and hence can penetrate the membrane, thus gaining access to the enzymes.

Aspartate being charged at physiological pH is a non-penetrant ion. It is transported in exchange for glutamate across the mitochondrial membrane (Azzi et al., 1967; LaNoue and Tischler, 1974). Aspartate aminotransferase activity was increased 3 times in mitoplasts due to activation by triton X-100 (Table 2). This reflected the inaccessibility of the enzyme to the substrate in untreated mitoplasts. In sonicated vesicles, the activity of aspartate aminotransferase was increased only 1.4 times due to activation by triton X-100 which reflected greater accessibility of the enzyme to the substrate on the inner side of the inner membrane. The membrane bound enzyme may, therefore be located on the inner side of the inner membrane.

Discussion

The present studies have shown that phosphate-dependent glutaminase, alanine and aspartate aminotransferase are not localized either in the outer membrane or the intermembrane space.

Most of the activity of alanine aminotransferases, PDG and glutamate dehydrogenase was found in the soluble fraction of broken mitoplasts (Table 1). Other workers found rat-liver PDG to have a loose attachment to the

Table 2. Comparative latency of enzyme activities in mitoplasts and sonicated vesicles of rat-liver mitochondria

	Cytochrome oxidase	β-hydroxybutyrate dehydrogenase	Alanine aminotransferase	Aspartate aminotransferase	*Glutaminase
		n moles substrate transformed/mg/min/mg protein			
Mitoplasts					
−activation	621 ± 53	35.1 ± 2.1	45.9 ± 4.4	456 ± 13	38.1
+activation	1,057 ± 133	47.9 ± 2.5	40.9 ± 4.1	1,371 ± 45	35.1
Sonicated vesicles					
−activation	847 ± 131	202 ± 11	4.3 ± 1.8	991 ± 22	3.9
+activation	2,837 ± 141	202 ± 11	4.3 ± 1.8	1,342 ± 52	3.9

The procedure for preparing mitoplasts and sonicated vesicles was as described in the methods section.
Each result that is followed by the standard deviation is the mean of four experiments. *, results represent the mean of two experiments.
Activations were carried out as follows: lmg lubrol/mg protein for cytochrome oxidase; 0.1% Triton X-100 for aspartate aminotransferase; five-second sonication for β-hydroxybutrate dehydrogenase, glutaminase and alanine aminotransferase.

inner-membrane (McGivan et al., 1980) whereas glutamate dehydrogenase was partially bound to the inner membrane in chicken-liver and pig-heart mitochondria (Elduque et al., 1982; Comte and Gautheron, 1978) with this partial binding being reversible in ox-liver mitochondria depending on the effectors present (Pour-Rahimi and Nemat-Gorgani, 1987). Although some of these three enzymes may loosely bind to the inner membrane in particular conditions, tissues or species, the present studies show that they are matrix enzymes in rat-liver mitochondria.

The dual localization of aspartate aminotransferase and malate dehydrogenase in the inner membrane (sonicated vesicles) and matrix (supernatant) was consistent with the findings of other workers (Elduque et al., 1982; Teller et al., 1990).

It has been shown that when mitochondria respire using exogenous glutamate as a substrate larger quantities of aspartate are synthesized whereas smaller amounts of aspartate are synthesized when the substrate is endogenous glutamate generated from glutamine hydrolysis (Kovačević, 1971; Masola et al., 1985). Kovačević (1971) suggested that aspartate aminotransferase had a favourable localization with respect to exogenous glutamate. It is suggested that because of the presence of the glutamate/aspartate on the inner membrane (Azzi et al., 1967; LaNoue and Tischler, 1974), the aspartate generated from exogenous glutamate and oxaloacetate by membrane bound aspartate aminotransferase is immediately transported out of the mitochondrion. Further, the aspartate synthesized by mitochondria using exogenous glutamate has been shown not to be in equilibrium with the total food of aspartate in the matrix (Duszynski et al., 1978). The presence of membrane associated malate dehydrogenase would thus facilitate channelling of oxaloacetate required by aspartate aminotransferase in malate-supported glutamate oxidation in mitochondria eliminating diffusion problems that would be encountered if oxaloacetate was generated deeper in the matrix by soluble malate dehydrogenase. In the current case, the synthesis of aspartate probably takes place on inner side of the inner membrane since membrane-bound aspartate aminotransferase is located on this side according to present studies and also according to other studies (Teller et al., 1990; Fahien and Teller, 1992). On the other hand glutamate generated inside the mitochondria through glutamine hydrolysis would not be readily accessible to membrane bound aspartate aminotransferase since glutaminase is a matrix enzyme. Accessibility would be limited by hindered diffusion due to the viscosity of the matrix. This glutamate may be preferentially metabolized by glutamate dehydrogenase which is largely a matrix enzyme according to the present studies. Aspartate synthesis would thus be reduced. This may explain why aminooxyacetate, an inhibitor of aminotransferases, has little effect on oxygen uptake when glutamine is the substrate and also why smaller quantities of aspartate are synthesized when this aminoacid is a substrate for respiring mitochondria (Kovačević, 1971; Evered and Masola, 1984; Masola et al., 1985).

Alanine aminotransferase in rat-liver mitochondria was found to be poised for alanine synthesis whereas the cytosolic enzyme was poised for

pyruvate formation (Groen et al., 1982). Lenartowicz and Wotjczak (1988) studied the significance of the alanine aminotransferase reaction and concluded that it contributed 77–97% of net production of tricaboxylic acid (TCA) cycle intermediates from glutamate in rat-liver mitochondria. There was also considerable synthesis of alanine from glutamate in rat-enterocyte mitochondria in the presence of pyruvate (Masola et al., 1985). All these results indicate that the function of the mitochondrial enzyme is the provision of TCA cycle intermediates from glutamate and with formation of alanine, a function that cannot be performed by the aspartate aminotransferase reaction since it consumes the TCA cycle intermediate, oxaloacetate. Alanine aminotransferase, a matrix enzyme according to the present results, is therefore conveniently located in the matrix for 2-oxoglutarate synthesis. Alanine synthesis from glutamine in the presence of pyruvate was 50% of that with glutamate as the substrate in rat enterocyte mitochondria (Masola et al., 1985). If phosphate-dependent glutaminase, which is a mitochondrial enzyme in this tissue (Pinkus and Windmueller, 1977) has a matrix localization like the liver enzyme, then there have to be other factors which limit alanine synthesis from glutamine and pyruvate. Large quantities of glutamate were synthesised from glutamine hydrolysis hence glutamate availability was not the limiting factor (Masola et al., 1985).

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