PROTEASE RESISTANCE OF ASPARTATE AMINOTRANSFERASE IMPORTED IN MITOCHONDRIA

E. MARRA, S. PASSARELLA, S. DOONAN+, E. QUAGLIARIELLO and C. SACCONE
Centro di Studio sui Mitocondri e Metabolismo Energetico, Istituto di Chimica Biologica, Università di Bari, 70126 Bari, Italy
and *+Department of Biochemistry, University College, Cork, Ireland*

Received 7 October 1980

1. Introduction

The majority of mitochondrial proteins are synthesized in the cell cytoplasm and subsequently transported to their sites of action in the mitochondrion. It is now established that synthesis and transport are not contemporaneous events, in distinction to the biosynthesis of secretory proteins, and that cytoplasmic pools of translation products exist. It appears that those products are in some cases precursors of the native proteins carrying N-terminal extension and in other cases not [1–3].

Aspects of the problem of mitochondrial biogenesis that are obscure at present include the mechanism by which cytoplasmically synthesized proteins are selectively transported into the organelle. To study this problem we have carried out in vitro experiments using the mitochondrial (mAAT) and the cytoplasmic (cAAT) isozymes of aspartate aminotransferase, a pair of structurally related proteins both of which are synthesized in the cytoplasm but which differ in their subcellular localization [4]. Using a variety of different experimental approaches we have shown that isolated mitochondria interact only with the mitochondrial isozyme, the nature of interaction depending on experimental conditions [5–8].

In a suspension of low ionic strength, the interaction is predominantly one of binding of mAAT to the outer surface of the mitochondria. At higher ionic strength, this external binding is abolished and uptake of the isozyme into the organelle could be demonstrated [6]. In none of the experimental systems cited above have we until now used one of the most widely accepted criteria for demonstration of sequestration of proteins in organelles, namely, resistance to proteases of the imported proteins [9]. The results of such experiments are reported here.

2. Materials and methods

Details of techniques used to demonstrate external binding or uptake of mAAT into mitochondria have been reported [5,6]. Variations in the conditions are given in the legends to the tables and figure. The sample of radiolabeled mAAT used here had an activity of 18 000 cpm/μg in the counting system of [6]. The proteases trypsin, chymotrypsin and pronase were purchased from Merck.

3. Results and discussion

The first experimental system used (table 1) involved incubation of organelles with native mAAT under conditions where the enzyme is either predominantly bound to the outer face of the mitochondria (10 mM Tris–HCl) or where uptake is the major event (20 mM Tris–HCl). The two processes are distinguished by measuring enzyme activity associated with the organelles after collection by centrifugation, either in the absence (externally bound enzyme) or presence (externally bound plus internal enzyme) of Triton X-100. Under conditions where only uptake is thought to occur, no enzyme activity could be detected externally bound after sedimentation of the mitochondria, but the activity measurable after lysis with Triton X-100 was higher than in the control where no mAAT had been added; the difference between the two assays represents enzyme

Table 1
Effect of pronase or trypsin + chymotrypsin on binding and uptake of native mAAT in rat liver mitochondria

	Total enzyme activity			
	10 mM Tris–HCl (binding)		20 mM Tris–HCl (uptake)	
	Pellet suspended without Triton	Pellet resuspended with Triton	Pellet suspended without Triton	Pellet resuspended with Triton
Control with mitochondria alone	–	4.95	–	4.83
Mitochondria + AAT Additions:				
None	1.15	6.42	0	5.5
Pronase	0	5.58	0	5.5
Trypsin + chymotrypsin	0	5.58	0	5.4

Rat liver mitochondria (2.1 mg protein) were incubated in a medium containing 0.25 M sucrose, 1 mM EGTA, 10 mM or 20 mM Tris–HCl (pH 7.3) in the presence of 14 μg mAAT (total enzyme activity = 6.4). The mixture was then incubated for 20 min, at 23°C with pronase (300 μg/ml) or with trypsin and chymotrypsin (250 μg of each/ml). After incubation, mitochondria were collected by centrifugation; enzyme assays were carried out on the pellet suspended in presence and absence of Triton X-100. Enzyme activity is expressed in arbitrary units

imported into the organelles (~10% of that added externally). When proteases were added to the incubation medium there was no decrease in the enzyme activity measurable after treatment with detergent showing that the extra enzyme activity compared with the control was inaccessible to protease attack. It should be noted that in control experiments not reported here in detail, 30–40% of mAAT was digested by the protease after incubation under the conditions in the table 1 as judged by loss of activity of the native isozyme or decrease in radioactivity precipitable by trichloroacetic acid in the case of the radiolabeled enzyme; this incomplete digestion is consistent with the known resistance to proteases of the native enzyme. The same percentage of digestion was found using higher concentrations of proteolytic enzymes (up to 600 μg/ml pronase, 500 μg/ml trypsin plus 500 μg/ml chymotrypsin). Moreover the same percentage of digestion was found with the endogenous enzyme, that is in a control experiment in which the mitochondria have been lysed with detergent before the addition of proteases at the same concentrations.

The results in table 1, under conditions favouring external binding of mAAT to mitochondria, are of particular interest. In the absence of proteases, ~20% of the added mAAT activity could be detected externally bound as judged by assay in the absence of Triton X-100. This externally bound enzyme was completely destroyed by proteases. Two points should be noted.

(i) All the externally bound enzyme was inactivated by proteases whereas incubation of the native mAAT with proteases under the same conditions resulted in only a 30–40% inactivation (see above); it is tempting to speculate that this increased susceptibility to proteolysis of mAAT bound to mitochondria, is the result of a conformational change consequent on binding.

(ii) After treatment with proteases, the total enzyme activity measurable in the presence of Triton X-100 was higher than that of the control mitochondria to which mAAT had not been added. This shows that under conditions favouring external binding of the isozyme, a substantial uptake into the mitochondria had also occurred. We were not able to demonstrate this previously with certainty because the large amount of externally bound enzyme obscured the effect [6].

The second system that we have devised to measure uptake of mAAT into mitochondria involves the use of radiolabeled enzyme. Protease resistance of imported mAAT has been examined using this system also (table 2). Again considering no first conditions where external binding is absent, the mitochondrial pellet after incubation with radiolabeled mAAT showed a radioactivity of 15 590 cpm, that is ~9% of the radioactivity added. This radioactivity was largely

Table 2
Effect of pronase or trypsin + chymotrypsin on binding and uptake of labeled mAAT in rat liver mitochondria

Additions	Radioactivity associated with mitochondria			
	10 mM Tris–HCl (binding)		20 mM Tris–HCl (uptake)	
	cpm	% control	cpm	% control
None	43 267	100	15 594	100
Pronase	12 547	29	13 410	86
Trypsin + chymotrypsin	13 845	32	12 787	82

Rat liver mitochondria (2.8 mg protein) were incubated in a medium containing 0.25 M sucrose, 1 mM EGTA, 10 mM or 20 mM Tris–HCl (pH 7.3) in the presence of 10 μg enzyme (18 000 cpm/μg). The mixture was then incubated for 20 min, at 23°C with pronase (300 μg/ml) or with trypsin and chymotrypsin (250 μg of each/ml). After incubation, mitochondria were collected by centrifugation and the radioactivity associated with the mitochondria was measured

insensitive to added proteases. The small decrease in measured radioactivity compared with the control was probably due to damage of the mitochondria on prolonged incubation with proteolytic enzymes and consequent leakage of the sequestered enzyme. Under conditions favouring external binding, 25% of the added radioactivity remained associated with the mitochondrial pellet after incubation without proteases. In the presence of proteases 70% of this radioactivity was found to be sensitive. By analogy with the results in table 1, we take this to mean that after incubation with isozyme in a medium containing 10 mM Tris–HCl buffer, 70% of the associated isozyme was externally bound to the mitochondria (protease-sensitive) and 30% had been taken up into the organelles (protease-resistant). It should be pointed out that in repeated experiments, the precise amount of uptake of enzyme and the ratio between uptake and binding varied, probably depending on variability in mitochondrial preparations, but the broad feature was always the same.

The last system employed was one which allows measurements of the amount of intramitochondrial AAT activity in intact mitochondria. In brief, mitochondria are incubated in an appropriate medium to which aspartate is added. After a time sufficient for the accumulation of this substrate, 2-oxoglutarate is added. Transport of this substrate into the organelles provides the complete substrate pair. Catalysis by AAT produces oxoaloacetate as one of the products and this is reduced by endogenous malate dehydrogenase with concomitant oxidation of NADH. This last process may be monitored by observation of a decrease in the fluorescence of NADH. We have previously presented detailed arguments in support of the view that this method provides a true measure of intramitochondrial AAT activity and that the increase of the rate of change of fluorescence observed on adding mAAT to the system is due to an uptake of the enzyme into the mitochondrial matrix [5]. The results in fig.1 show that this increase in rate of

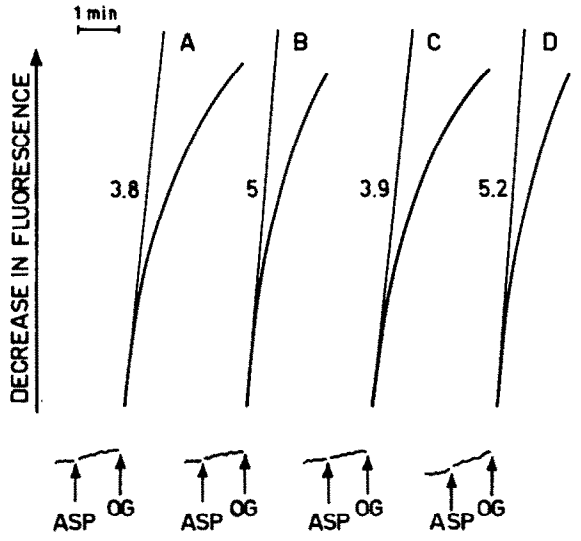

Fig.1. Insensitivity of incorporated enzyme to pronase. The mitochondria (2.1 mg protein) were preincubated for 20 min either with no additions (A), with 270 μg pronase/ml (C), with 8 μg mAAT (B) or with 8 μg mAAT followed 1 min later by 270 μg pronase/ml (D). Substrates were then added in the sequence shown and the resulting fluorescence changes were measured.

change of fluorescence is insensitive to pronase. In (A) and (B) mitochondria were incubated at 23°C for 20 min either in the absence or presence of pronase before measurements of the rate of change of fluorescence; no effect of pronase could be observed. In (C) mAAT was added at the beginning of the incubation period. After 20 min, the rate of change of fluorescence was measured and was 31% greater than that of the controls. In (D) pronase (270 μg/ml) was added 1 min after mAAT and again the rate of change of fluorescence was found to be 33% greater than in the controls; that is, enzyme sequestered in the mitochondria was insensitive to proteases. As a check on efficiency of proteolysis and on the integrity of the mitochondria after incubation with pronase, mitochondria were removed from each incubation mixture at the end of experiment and the supernatants tested for AAT activity. No activity was found in (A) or (C) showing that no leakage of endogenous enzyme had occurred. Comparison of (B) and (D) shows that the remaining external AAT was inactivated by 30% by action of the pronase showing that pronase was effective in partially digesting the added mAAT. In summary, using three different experimental systems, it has been shown that mAAT added to suspension of mitochondria is taken up into the organelles as judged by protease resistance. It has also been shown that, whereas native mAAT is only partially digested by proteases under the conditions used here, enzyme bound to the outer surface of the mitochondria is completely digested. The increased sensitivity to proteases on binding is suggestive of a conformational change. Hence as a working hypothesis, it may be proposed that the sequence of events involved in import of mAAT into mitochondria may be interaction with the mitochondrial surface accompanied by a conformational change, passage through the membrane system and then reversion to the native state. Whether these events also involve dissociation of the dimeric enzyme into monomers remains to be established, as indeed do other molecular aspects of the process; knowledge in this area is limited so far to the fact that blockage of a single reactive sulphydryl groups in mAAT prevents uptake into mitochondria [7].

References

[1] Chua, N. H. and Schmidt, G. W. (1979) J. Cell Biol. 81, 461–483.
[2] Schatz, G. (1979) FEBS Lett. 103, 203–211.
[3] Harmey, M. A., Hallermayer, G., Korb, H. and Neupert, W. (1977) Eur. J. Biochem. 81, 533–544.
[4] Mahler, H. R. and Davidowicz, K. (1973) Proc. Natl. Acad. Sci. USA 70, 111–114.
[5] Marra, E., Doonan, S., Saccone, C. and Quagliariello, E. (1977) Biochem. J. 164, 685–691.
[6] Marra, E., Doonan, S., Saccone, C. and Quagliariello, E. (1978) Eur. J. Biochem. 83, 427–435.
[7] Marra, E., Passarella, S., Doonan, S., Saccone, C. and Quagliariello, E. (1979) Arch. Biochem. Biophys. 195, 269–279.
[8] Saccone, C., Marra, E., Passarella, S., Doonan, S. and Quagliariello, E. (1979) in: Membrane Bioenergetics (Lee, C. P. et al. eds) pp. 153–164, Addison-Wesley, Reading, MA.
[9] Maccecchini, M. L., Rudin, Y., Blobel, G. and Schatz, G. (1979) Proc. Natl. Acad. Sci. USA 76, 343–347.