Removal of an *N*-terminal peptide from mitochondrial aspartate aminotransferase abolishes its interactions with mitochondria *in vitro*

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(Received 19 November 1984/8 February 1985; accepted 4 March 1985)

Treatment of mitochondrial aspartate aminotransferase from rat liver with trypsin leads to specific cleavage of the bonds between residues 26 and 27, and residues 31 and 32. The proteolysed enzyme has only a small residual catalytic activity, but retains a conformation similar to that of the native form as judged by accessibility and reactivity of cysteine residues. Proteolysis abolishes the ability of the enzyme either to bind to mitochondria or to be imported into the organelles. This suggests that the *N*-terminal segment of the native enzyme is essential for both of these functions, at least in the model system used to study the import process.

Most mitochondrial proteins are synthesized in the cell cytosol in the form of precursors of M_r values greater than those of the mature proteins and are subsequently imported into the organelles (for a review see Doonan *et al.*, 1984). We have shown, however, that mature aspartate aminotransferase (EC 2.6.1.1) (Marra *et al.*, 1977) and malate dehydrogenase (EC 1.1.1.37) (Passarella *et al.*, 1980) are imported into mitochondria *in vitro*. In the former case the phenomena observed are either import alone, or import accompanied by external binding of enzyme to the mitochondria, depending on the experimental conditions (Marra *et al.*, 1978, 1980).

The uptake of these mature proteins into mitochondria is perhaps surprising, since both aspartate aminotransferase (Sonderegger *et al.*, 1980; Sakakibara *et al.*, 1980; Sannia *et al.*, 1982) and malate dehydrogenase (Aziz *et al.*, 1981) are now known to be synthesized in precursor form. Indeed there have been claims (reviewed by Doonan *et al.*, 1984) that mature proteins are not imported into mitochondria under the experimental conditions employed by other workers. Uptake of mature aspartate aminotransferase and malate dehydrogenase in our model system is, however, similar to the process occurring *in vivo* in terms of the rate of import, involvement of membrane receptors, and dependence on a transmembrane electrical potential (Doonan *et al.*, 1984). Hence, although the import of these mature

proteins is not a physiological process, we maintain that it accurately reflects some aspects of the process occurring *in vivo*, and hence that studies of the effects of chemical modifications on the uptake of mature protein can yield useful information on the physiological process.

A notable feature of the structure of mitochondrial aspartate aminotransferase is that the *N*-terminal amino acid sequence of the enzyme has been very highly conserved during evolution, whereas that of the cytosolic isoenzyme has not (Bossa *et al.*, 1981). One reason for a higher degree of conservation of regions of the structure of the mitochondrial isoenzyme compared with the cytosolic form might be that these regions are specifically involved in transport through the mitochondrial membrane system. In the case of the *N*-terminal sequence this possibility can be directly tested, since it has been shown that, for the enzyme from chicken heart, this part of the protein can be specifically removed by treatment with trypsin (Sandmeier & Christen, 1980).

We show here that the *N*-terminal sequence of the enzyme from rat liver can be similarly removed with little effect on the conformation of the enzyme. Furthermore, the proteolysed enzyme so produced neither binds to the outside of rat liver mitochondria nor is it imported into the organelles. The results suggest that the *N*-terminal section of the native enzyme is directly involved in the process of transport through the mitochondrial

membrane system. These results have been communicated in preliminary form (O'Donovan *et al.*, 1984).

Materials and methods

Materials

Rat liver mitochondrial aspartate aminotransferase was purified as described by Porter *et al.* (1981). Rat liver mitochondria were isolated as described by Marra *et al.* (1977) and suspended in a medium containing 0.25M-sucrose, 20mM-Tris/HCl, 1mM-EGTA, pH 7.4, at a protein concentration of about 40mg/ml. For measurements of external binding the Tris/HCl concentration in the medium was reduced to 10mM.

Trypsin (type XI) and 5,5'-dithiobis-(2-nitrobenzoic acid) were from Sigma Chemical Co., Poole, Dorset, U.K., Carboxypeptidase Y was from Pierce and Warriner (U.K.), Chester, Cheshire, U.K. 4-(*NN*-Dimethylamino)azobenzene 4'-isothiocyanate was from Fluka AG, Buchs, Switzerland, and 4-(*NN*-dimethylamino)azobenzene 4'-thiohydantoin derivatives of amino acids were synthesized as described by Wilson *et al.* (1979). 3-Aminopropyl-glass and *p*-phenylenedi-isothiocyanate were from LKB Biochrom, Cambridge, U.K. NaB³H₄ was from The Radiochemical Centre, Amersham, Bucks., U.K.

Limited proteolysis of aspartate aminotransferase

This was done essentially as described by Sandmeier & Christen (1980). Samples of trypsin (0.8mg in 10 μ l of 1mM-HCl) were added every 15min to 8.0mg of enzyme in 2ml of 50mM-Tris/HCl buffer, pH 8.9, at 37°C. Aliquots were taken every 1h and assayed for aspartate aminotransferase activity (Porter *et al.*, 1981). After 4h the sample was passed through a column (1.5cm $\times$ 30cm) of Sephadex G-75 equilibrated with sodium phosphate buffer (50mM, pH 7.4).

N-Terminal analysis

This was done by dansylation in the presence of sodium dodecyl sulphate as described by Gray (1972).

N-Terminal sequence analysis

Proteolysed aspartate aminotransferase (2mg) was coupled to 100mg of 3-aminopropyl-glass previously activated with *p*-phenylenedi-isothiocyanate as described in the LKB Solid Phase Sequencing Handbook. The solvent for coupling was 50% (v/v) pyridine containing 0.1% (w/v) sodium dodecyl sulphate.

An LKB Solid Phase Sequencer was converted

for use with 4-(*NN*-dimethylamino)azobenzene 4'-isothiocyanate as described by Hughes *et al.* (1979). The thiazolinone derivatives were converted by treating with HCl (200 μ l of 1M) at 80°C for 10min. Product 4-(*NN*-dimethylamino)azobenzene 4'-thiohydantoin derivatives were identified by t.l.c. as described by Wilson *et al.* (1979).

C-Terminal sequence analysis

Proteolysed enzyme in 0.1M-Mops buffer, pH 6.3, containing sodium dodecyl sulphate (0.1%) was treated with carboxypeptidase Y (1:50, w/w). Amino acids liberated after 40min of digestion at room temperature were identified by using a Locarte Auto Analyzer. Carboxypeptidase Y was incubated under the same conditions but in the absence of aspartate aminotransferase as a control.

Reaction with 5,5'-dithiobis-(2-nitrobenzoic acid)

Excess reagent was added to 0.5mg of native or proteolysed enzyme in 1ml of 50mM-sodium phosphate buffer, pH 7.4. The A_{412} was monitored as a function of time and the extent of reaction calculated by using a molar absorption coefficient of 13600M⁻¹·cm⁻¹ for the products (Ellman, 1959).

Radiolabelling of native and proteolysed enzyme

Enzymes (5mg/ml) in 20mM-triethanolamine/HCl buffer, pH 7.5, were treated with NaB³H₄ (10 μ l, 1Ci/ml, 4.7Ci/mmol) for 30min at room temperature. Pyridoxal 5'-phosphate was then added to 10 μ M, followed by an excess of unlabelled NaBH₄. Reagents were removed by gel filtration through a column (1cm $\times$ 25cm) of Sephadex G-25 equilibrated in 10mM-Tris/HCl buffer, pH 7.8. Enzymic assay showed that reduction of the cofactor-apoenzyme linkage was complete.

Measurement of incorporation of enzymes into mitochondria

This was done essentially as described by Marra *et al.* (1978). Mitochondria (approx. 2mg of protein) were incubated in 1ml of 0.25M-sucrose/1mM-EGTA/20mM-Tris/HCl, pH 7.4. Enzyme (10–80 μ g) was added and after 1min the organelles were collected by centrifugation. Supernatant solution was removed and the pellets were lysed by addition of Triton X-100 (100 μ l of 10%, w/v) followed by water (100 μ l). Insoluble material was removed by centrifugation and radioactivity in the clarified solution was determined. For measurement of external binding of enzyme to the organelles, 10mM-Tris/HCl buffer was used in the incubation medium, but otherwise the procedure was the same.

Results

Limited tryptic cleavage of mitochondrial aspartate aminotransferase from rat liver

Treatment of the enzyme with trypsin led to a progressive decrease in catalytic activity (Fig. 1). After 4 h the activity was about 4% of the starting value and did not decrease further when the period of digestion was extended. Very high trypsin/substrate ratios were required to achieve complete proteolysis, as was previously reported by Sandmeier & Christen (1980). Low- M_r material was removed by gel filtration, after which electrophoresis on a 5–20% gradient polyacrylamide gel in the presence of sodium dodecyl sulphate produced a single band with mobility slightly greater than that of the native enzyme (results not shown).

N-Terminal analysis of the proteolysed enzyme in the presence of sodium dodecyl sulphate (Gray, 1972) revealed two terminal residues, aspartic acid and lysine, in roughly equal amounts. There was no trace of serine, the *N*-terminal residue of the native enzyme (Huynh *et al.*, 1980; Bossa *et al.*, 1981).

The proteolysed enzyme was coupled to 3-aminopropyl-glass by using *p*-phenylenedi-isothiocyanate and sequence analysis was carried out with an LKB Solid Phase Sequencer modified for high-

Fig. 1. Decrease in catalytic activity of mitochondrial aspartate aminotransferase on limited proteolysis with trypsin

Samples of trypsin (0.8 mg) were added every 15 min to 8 mg of enzyme and aliquots were taken at the times shown for measurements of catalytic activity (●). As a control, enzyme was incubated under identical conditions but without addition of trypsin (■).

sensitivity sequencing with 4-(*NN*-dimethylamino)azobenzene 4'-isothiocyanate (Hughes *et al.*, 1979). Residues identified after each cycle of degradation are listed in Table 1. Identification was by t.l.c. and hence yields could not be determined. However, in the case where two residues were identified (cycle 4), the intensities of the spots were roughly equal.

The *N*-terminal amino acid sequence of the native enzyme is shown in Fig. 2; it should be noted that Huynh *et al.* (1980) reported a glycine residue at position 13, but Bossa *et al.* (1981) found proline at this position, as in all other mitochondrial aspartate aminotransferases so far examined. From the results obtained by *N*-terminal analysis and from sequence analysis (Table 1) it appears that cleavage by trypsin had occurred between residues 26 and 27, and between residues 31 and 32. No native enzyme remained; this was shown by the absence of tryptophan, which is particularly easy to detect, at cycles 3 and 4 of the degradation.

A sample of the proteolysed enzyme was treated with carboxypeptidase Y and the liberated amino acids were analysed. The residues released (mol/

Table 1. Amino acid residues identified by solid-phase sequence analysis of proteolysed aspartate aminotransferase

Cycle	Residues
1	—*
2	Met†
3	Asn
4	Leu, Ser
5	Gly‡

* Residues at cycle 1 remain bound to the solid support.

† The thin-layer plate was run only in the first solvent for identification of non-polar residues.

‡ The lysine residue expected at this position (Fig. 2) would have remained bound to the solid support.

Fig. 2. *N*-Terminal sequence of rat liver mitochondrial aspartate aminotransferase (Huynh *et al.*, 1980; Bossa *et al.*, 1981) showing positions of cleavage by trypsin. The numbering system used is that of the homologous cytosolic isoenzyme (Barra *et al.*, 1980); on this system the *N*-terminal residue of the mitochondrial form is residue 3.

mol of protein) were lysine (0.7), threonine (0.3) and valine (0.2). These results were consistent with the C-terminal sequence (Val-Thr-Lys) of the native enzyme (Huynh *et al.*, 1980) and showed that no proteolysis had occurred at the C-terminal end of the protein.

Reactivities of cysteine residues in native and proteolysed aspartate aminotransferase

Both forms of the enzyme were treated with 5,5'-dithiobis-(2-nitrobenzoic acid), and the extent and rate of reaction measured from the change in A_{412} . The relative rates of reaction of the two enzymes in the presence of substrates (0.4 mM-oxaloacetate and 11 mM-aspartate) were also determined. The results are given in Table 2.

Failure of proteolysed aspartate aminotransferase to interact with mitochondria

Both native and proteolysed aspartate aminotransferases were radiolabelled by reduction of the apoenzyme-cofactor linkage with NaB^3H_4 . Radioactivities of the reduced forms were 6.8×10^6 c.p.m./mg and 3.5×10^6 c.p.m./mg for native and proteolysed enzyme respectively. Increasing amounts of radiolabelled enzymes were incubated with mitochondria under conditions (see the Materials and methods section) which promote uptake of native aspartate aminotransferase into the organelles (Marra *et al.*, 1978); the enzyme imported becomes resistant to proteinases (Marra *et al.*, 1980). Mitochondria were recovered by centrifugation, lysed, and radioactivity associated with the pellets was measured.

The results are given in Fig. 3. The native enzyme showed a progressive increase in uptake with amount of externally added protein. At higher levels (above 50 $\mu\text{g}/\text{ml}$) the uptake reached a constant value of about 9 μg of enzyme imported (results not shown). The results with proteolysed enzyme were completely different (Fig. 3). Up to the maximum concentration used (20 $\mu\text{g}/\text{ml}$) less than 2% of the added enzyme was associated with the mitochondrial pellet. We have shown previously (Marra *et al.*, 1978) that this level of association results from physical entrapment of incubation medium in the pellet. Hence, allowing

for this effect, the results in Fig. 3 indicate that uptake of proteolysed enzyme did not occur.

It is known that there is a receptor in the mitochondrial membrane system responsible for import of aspartate aminotransferase (Marra *et al.*, 1982; Passarella *et al.*, 1982). The results quoted above did not preclude the possibility that proteolysed enzyme can bind to this receptor but that subsequent import is blocked. This possibility was tested by measuring import of labelled native enzyme (15 μg) into mitochondria (2 mg of protein) in the presence or absence of unlabelled proteolysed enzyme (19.5 μg). The amount of native enzyme imported was 0.95 μg in the absence of the proteolysed form, and was 1.00 μg and 1.04 μg respectively when added together with, or 1 min

Fig. 3. Association of native or proteolysed aspartate aminotransferase with mitochondrial pellets. Various amounts of radiolabelled native (●) or proteolysed (■) enzyme were incubated with mitochondria for 1 min in a medium containing 250 mM-sucrose, 1 mM-EGTA and 20 mM-Tris/HCl, pH 7.4. Mitochondria were collected by centrifugation, lysed, and associated radioactivity was determined.

Table 2. Reaction of native and of proteolysed aspartate aminotransferase with 5,5'-dithiobis-(2-nitrobenzoic acid). For the experiments reported in the last column, substrate concentrations used were 0.4 mM-oxaloacetate and 11 mM-aspartate.

Enzyme	Available SH groups (mol/mol)	Second-order rate constant ($\text{M}^{-1} \cdot \text{min}^{-1}$)	Increase in rate in presence of substrates (-fold)
Native	1.1	46	2.5
Proteolysed	1.2	53	1.4

after, the proteolysed enzyme. Binding of proteolysed enzyme to the receptors would have decreased uptake of the native enzyme, contrary to the results obtained.

We have also shown previously (Marra *et al.*, 1978) that when mitochondria are incubated with aspartate aminotransferase in a medium containing 10mM-Tris/HCl in place of the more concentrated buffer, then a substantial amount of the enzyme binds to the outside of the organelles. Externally bound enzyme could be distinguished from imported enzyme by sensitivity to proteinases of the former and resistance to proteinases of the latter (Marra *et al.*, 1980). The behaviour of the proteolysed enzyme under conditions favouring external binding (see the Materials and methods section) was investigated. Quantities of radio-labelled proteolysed enzyme in the range 4.9–19.6 μ g were added to mitochondria (2.0mg of protein) in 1 ml of incubation medium. After recovery of the organelles, less than 2% of the added radioactivity was found to be associated with the mitochondria; as argued above, this is the quantity expected to be physically entrapped in the pellets, and hence the results show external binding of proteolysed enzyme to be negligible. Under the conditions used, 30–40% of added native enzyme binds externally (Marra *et al.*, 1978).

Discussion

Cleavage of the rat liver aspartate aminotransferase with trypsin occurred specifically between residues 26 and 27, and between residues 31 and 32, exactly as with the enzyme from chicken heart (Sandmeier & Christen, 1980). The similarity in behaviour of the enzyme from the two sources is not surprising, since, with the exception of residue 5, the isoenzymes have identical sequences up to residue 50 (Graf-Hausner *et al.*, 1983). No other peptide bonds were hydrolysed. The proteolysed enzyme appeared to have a three-dimensional structure similar to that of the native form in that only one cysteine residue out of a total of seven (Huynh *et al.*, 1980) was available for reaction with 5,5'-dithiobis-(2-nitrobenzoic acid), and the reactivity of this cysteine was very similar in the two forms of the enzyme (Table 2). Reactivities of cysteines are recognised to be sensitive indicators of conformation in aspartate aminotransferases (Petrilli *et al.*, 1981). On the other hand, the increase in reactivity of the exposed cysteine in the presence of substrates (Gehring & Christen, 1978) was less for the proteolysed enzyme compared with the native form. This is indicative of some changes in conformational mobility in the active-site region.

The results reported here show that, in contrast

to native aspartate aminotransferase, the proteolysed enzyme neither enters mitochondria nor binds to them externally. The latter observation is of interest, since external binding in media of low ionic strength could in principle be a non-specific electrostatic interaction between the positively charged isoenzyme (Barra *et al.*, 1980) and the negatively charged outer membrane. It should be noted, however, that the pieces of sequence removed by trypsin contain about 13% of basic residues, whereas the enzyme as a whole contains about 14% of such residues (Huynh *et al.*, 1980); thus proteolysis does not decrease the basicity of the enzyme. This suggests that external binding is a specific effect, not related solely to basicity, and that externally bound enzyme may be on the pathway to import.

The results further suggest that the *N*-terminal section of the native protein is directly involved in binding to the membrane receptor which mediates the uptake process in our model system (Marra *et al.*, 1982; Pasarella *et al.*, 1982). This would be consistent with the high degree of conservation of this part of the structure of mitochondrial aspartate aminotransferases as compared with that of the cytosolic forms (Bossa *et al.*, 1981). We have previously shown (Marra *et al.*, 1979) that blockage of the single reactive cysteine (probably at position 166) also inhibits uptake of the enzyme into mitochondria. Thus the picture that emerges of the uptake process is one of interaction between the membrane receptor and multiple sites in the structure of aspartate aminotransferase, the *N*-terminus providing one such site. The extent to which this is true also *in vivo*, where the enzyme transported has an *N*-terminal precursor region, remains to be determined.

K. M. C. O'D. thanks the Department of Education for financial support and the European Molecular Biology Organization for a short-term fellowship.

References

- Aziz, L. E., Chien, S.-M., Patel, H. V. & Freeman, K. B. (1981) *FEBS Lett.* **133**, 127–129
- Barra, D., Bossa, F., Doonan, S., Fahmy, H. M. A., Hughes, G. J., Martini, F., Petruzzelli, R. & Wittmann-Liebold, B. (1980) *Eur. J. Biochem.* **108**, 405–414
- Bossa, F., Barra, D., Martini, F., Schininà, E., Doonan, S. & O'Donovan, K. M. C. (1981) *Comp. Biochem. Physiol.* **69B**, 753–760
- Doonan, S., Marra, E., Passarella, S., Saccone, C. & Quagliariello, E. (1984) *Int. Rev. Cytol.* **91**, 141–186
- Ellman, G. L. (1959) *Arch. Biochem. Biophys.* **82**, 70–77
- Gehring, H. & Christen, P. (1978) *J. Biol. Chem.* **253**, 3158–3163
- Graf-Hausner, U., Wilson, K. J. & Christen, P. (1983) *J. Biol. Chem.* **258**, 8813–8826
- Gray, W. R. (1972) *Methods Enzymol.* **25**, 121–138

- Hughes, G. J., Winterhalter, K. H., Lutz, H. & Wilson, K. J. (1979) *FEBS Lett.* **108**, 92-97
- Huynh, Q. K., Sakakibara, R., Watanabe, T. & Wada, H. (1980) *Biochem. Biophys. Res. Commun.* **97**, 474-479
- Marra, E., Doonan, S., Saccone, C. & Quagliariello, E. (1977) *Biochem. J.* **164**, 685-691
- Marra, E., Doonan, S., Saccone, C. & Quagliariello, E. (1978) *Eur. J. Biochem.* **83**, 427-435
- Marra, E., Passarella, S., Doonan, S., Saccone, C. & Quagliariello, E. (1979) *Arch. Biochem. Biophys.* **195**, 269-279
- Marra, E., Passarella, S., Doonan, S., Quagliariello, E. & Saccone, C. (1980) *FEBS Lett.* **122**, 33-36
- Marra, E., Passarella, S., Doonan, S., Casamassima, E., Quagliariello, E. & Saccone, C. (1982) *Ital. J. Biochem.* **31**, 289-290
- O'Donovan, K. M. C., Doonan, S., Marra, E. & Passarella, S. (1984) *Biochem. Soc. Trans.* **12**, 444-445
- Passarella, S., Marra, E., Doonan, S. & Quagliariello, E. (1980) *Biochem. J.* **192**, 649-658
- Passarella, S., Marra, E., Doonan, S., Perlino, E., Saccone, C. & Quagliariello, E. (1982) *Ital. J. Biochem.* **31**, 291-292
- Petrilli, P., Pucci, P., Garzillo, A. M., Sannia, G. & Marino, G. (1981) *Mol. Cell. Biochem.* **35**, 121-128
- Porter, P. B., Barra, D., Bossa, F., Cantalupo, G., Doonan, S., Martini, F., Sheehan, D. & Wilkinson, S. M. (1981) *Comp. Biochem. Physiol.* **69B**, 737-746
- Sakakibara, R., Huynh, Q. K., Nishida, Y., Watanabe, T. & Wada, H. (1980) *Biochem. Biophys. Res. Commun.* **95**, 1781-1788
- Sandmeier, E. & Christen, P. (1980) *J. Biol. Chem.* **255**, 10284-10289
- Sannia, G., Abrescia, P., Colombo, M., Giardina, P. & Marino, G. (1982) *Biochem. Biophys. Res. Commun.* **105**, 444-449
- Sonderegger, P., Jaussi, R. & Christen, P. (1980) *Biochem. Biophys. Res. Commun.* **94**, 1256-1260
- Wilson, K., Hunziker, P. & Hughes, G. J. (1979) *FEBS Lett.* **108**, 98-102