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IN VITRO SYNTHESIS OF PRECURSOR FORMS OF PIG HEART ASPARTATE AMINOTRANSFERASE ISOZYMES*

G. Sannia, P. Abrescia, M. Colombo, P. Giardina & G. Marino

Istituto di Chimica Organica e Biologica, Università di Napoli
Via Mezzocannone 16, I-80134 Napoli (Italy).

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Precursor forms of the isozymes of aspartate aminotransferase from pig heart were synthesized *in vitro* and purified by binding to specific antibodies. Analysis by sodium dodecylsulfate polyacrylamide gel electrophoresis showed that the precursor of the cytosolic enzyme has a similar molecular weight to that of the mature protein whereas the precursor of the mitochondrial isozyme has a molecular weight greater than that of the corresponding mature protein ($\Delta MW \cong 2500$). Preliminary sequence studies seem to suggest that the precursor of the mitochondrial isozyme has an extra N-terminal peptide sequence while that of the cytosolic protein has only an extra N-terminal methionine residue.

Aspartate aminotransferase (E.C. 2.6.1.1.) exists in the eukaryotic cells as two distinct isozymes, one located in the cytosol and the other in the mitochondria, but both encoded by the nuclear genome (1).

The mechanism of the import of mitochondrial AAT into the organelle is under debate (2,3) in fact mature protein is specifically incorporated *in vitro* into mitochondria (4,5) and precursors of higher molecular weight than the mature forms occur in chicken heart (6) and in rat liver (7).

Comparative work could help to ascertain the primary structure relationships among precursors within different species and may throw light on the basic structural requirements of the two different genes and the factors that determine the selective import of one isozyme, but not the other, into the organelle.

We have undertaken a study of the *in vitro* synthesis of the two aspartate aminotransferase isozymes from pig heart. The primary structures of these two

Abbreviations: cAAT and mAAT, cytosolic and mitochondrial aspartate aminotransferase, respectively; the prefix p stands for precursor; SDS, sodium dodecylsulfate.

* Dedicated to Prof. A.E. Braunstein on the occasion of his 80th birthday.

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isozymes have been described (8-11) and their tertiary structures are under investigation (12,13).

In this paper we report on the *in vitro* translation of pig heart polyadenylated RNA which codes for the synthesis of putative precursor forms of both cytosolic and mitochondrial aspartate aminotransferases.

MATERIALS AND METHODS

Preparation of labelled AAT isozymes. Pig heart aspartate aminotransferases were purified to homogeneity as described previously (14). Radioactively labelled isozymes were prepared by reduction of the aldimine bond of pyridoxal-5'-phosphate in the holoenzymes using ($^3H_4$)-borohydride (10 Ci/mmol, the Radiochemical Centre, Amersham).

Preparation of antibodies. Antibodies showing specific immunoreactivity toward the pig heart AAT isozymes were produced in two rabbits. 100 µg of protein per kg body weight in 50 µl of physiological solution were emulsified with an equal volume of complete Freund's adjuvant and injected into the rabbits. Injections were repeated every 20 days for three months. Bleeding was performed 10 days after the last injection. The antisera obtained were stored at -20°C and their specificity was determined as described in the text (Fig.1).

Isolation of polyadenylated RNA. Fresh pig heart was frozen at -70°C and homogenized in 8 vols. of buffer containing 50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 0.5% SDS, 0.1% heparin and 0.1% diethyl pyrocarbonate (pH 7.5). After three phenol-chloroform extractions followed by precipitation in 70% cold ethanol, the RNA fraction was isolated by overnight precipitation in 2 M LiCl at 4°C. Polyadenylated RNA was purified from total RNA by affinity chromatography on oligo-(dT)-cellulose (15) or poly(U)-Sepharose (16).

In vitro protein synthesis. Polyadenylated RNA was incubated in a rabbit reticulocyte lysate (nuclease treated, message dependent) in the presence of (^{35}S)methionine (1000 Ci/mmol, 1µCi/µl) alone or together with either L- 2,3-^3H alanine (53 Ci/mmol, 1µCi/µl) or L-(3-^3H)serine (17 Ci/mmol, 1µCi/µl).The radioactive amino acids and reticulocyte lysate were from the Radiochemical Centre (Amersham).

Immunoprecipitation and SDS-polyacrylamide gel electrophoresis. 5 vols. of a buffer 50 mM Tris-HCl containing 150 mM NaCl, 5 mM EDTA, 1mM Triton (pH 7.2) were added to the incubation mixture. After centrifugation (100,000 x g, 0°C, 90 min) 100 µl of 20% protein A-Sepharose Cl-4B (Pharmacia) were added and incubated at 4 °C for 1 h. After further centrifugation the supernatant was divided into two aliquots and mixed with 40 µg of either anti-mAAT or anti-cAAT antibodies. Mixtures were incubated overnight at 4°C and then 100 µl of a 20% suspension of protein A-Sepharose were added to each tube. Two hours later the immunoprecipitated samples were collected by centrifugation,washed 8 times with the immunoprecipitation buffer, resuspended in 50 µl of electrophoresis buffer (60 mM Tris-HCl, 5% SDS, 10% glycerol, 5% mercaptoethanol,pH 7.5) and held 3 min at 100°C. The suspensions were centrifuged and the resulting supernatants were analyzed by electrophoresis on 9-15% polyacrylamide slab gel in 0.1% SDS (17). Gel treatment for fluorography was performed according to Bonner and Laskey (18), using Kodak-X-Omat XRP-1 films. In separate experiments, immunoprecipitated samples were lyophilized, redissolved in 300 µl of trifluoroacetic acid, and used for sequence determinations.

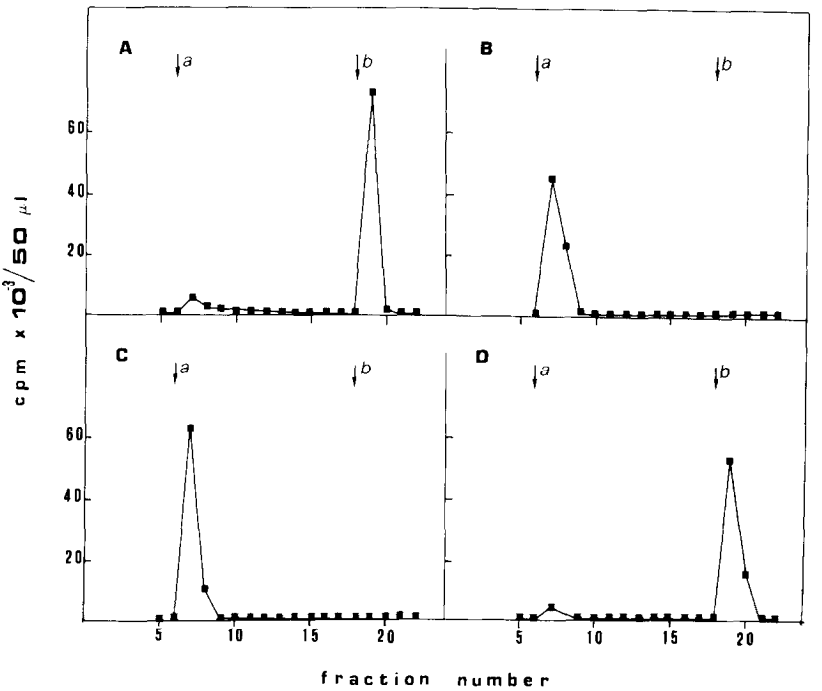

Fig.1 - Specificity of anti-AAT antibodies. Affinity chromatography performed on columns of Sepharose-protein A loaded first with 100 µl of antisera anti-mAAT (*A,B*) or anti-cAAT (*C,D*) and then with samples of (^{3}H)-mAAT (*A,C*) or (^{3}H)-cAAT (*B,D*). Diagrams report ^{3}H radioactivity of effluents: ↓a) loading of the enzyme; ↓b) elution with 0.1 M glycine-HCl pH 3.0, in conditions of dissociation of antibodies--protein A complexes

Sequence determination. 3 mg ovalbumin was added to the immunoprecipitated samples to act as a carrier (19) for manual Edman degradations. The radioactivity of the thiazolinone derivative was measured in a liquid scintillation counter (Beckman LS 8100) set up for double label counting.

RESULTS AND DISCUSSION

Specific anti-mAAT or anti-cAAT antibodies were obtained from the serum of rabbits immunized with pure pig heart isozymes. Their specificity was tested by affinity chromatography (Fig.1), and the results show that a Sepharose-protein A column loaded with anti-cAAT antibodies does not bind any detectable amount of mature mAAT and viceversa.

Polyadenylated RNA fractions (on average 1.3% of the total RNA), purified from pig heart tissue, were tested for their ability to stimulate *in vitro* protein synthesis. After translation experiments in the presence of (^{35}S)-methionine an average of 3.9×10^7 cpm per ml of lysate, containing 2.4 A_{260} units of polyadenylated RNA, were recovered upon precipitation with trichloroacetic acid.

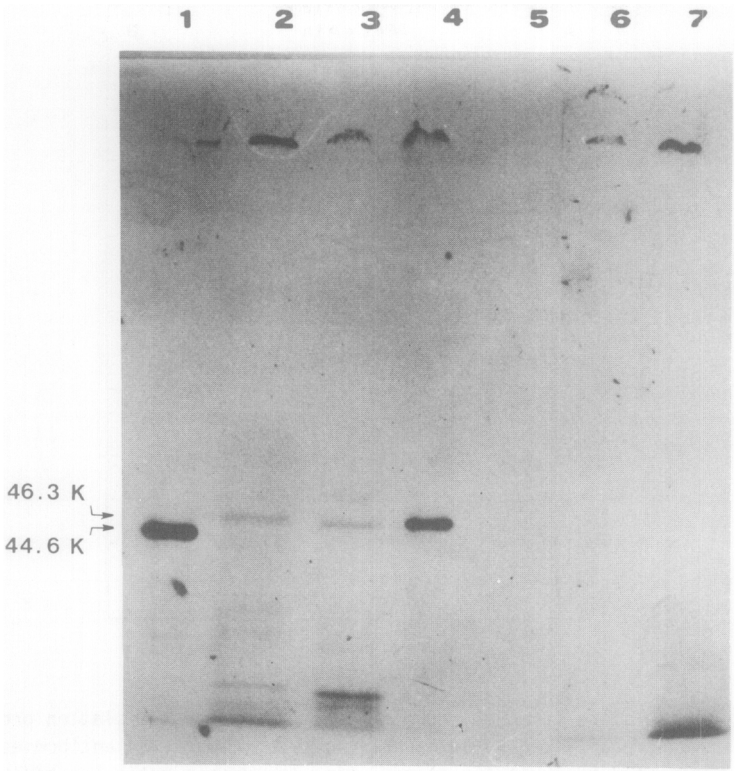

Fig.2 - SDS-gel electrophoresis of the *in vitro* translation products. Lane 1: (^{3}H)-mAAT (44.7 kdaltons); lane 2: translation products recognized by anti-mAAT; lane 3: translation products recognized by anti-cAAT; lane 4: (^{3}H)-cAAT (46.3 kdaltons); lane 5: translation products obtained with no mRNA added; lanes 6 and 7: translation products immunoprecipitated by antisera anti-cAAT (lane 6) or anti-mAAT (lane 7) in the presence of an excess of cAAT and mAAT respectively.

Translation products recognized by anti-cAAT or anti-mAAT antibodies were isolated by precipitation of the complexes with protein A-Sepharose; the radioactivity recoveries were 0.80% and 0.57% respectively.

On SDS-gel electrophoresis (Fig.2) it can be observed that: i) the translation products immunoprecipitated by antibodies anti-cAAT show a band having a mobility comparable with that of cAAT and disappearing when immunoprecipitation was carried out in the presence of cAAT excess; ii) the translation products immunoprecipitated with anti-mAAT antibodies show a band having a mobility lower than that of mAAT and disappearing when immunoprecipitation was carried out in the presence of mAAT excess. Therefore the newly synthesized polypeptides can be considered putative precursors of the respective mature proteins.

The molecular weight of the precursor of cAAT (p-cAAT) was similar to that of cAAT, within the gel resolution limit (± 400), while the precursor of mAAT

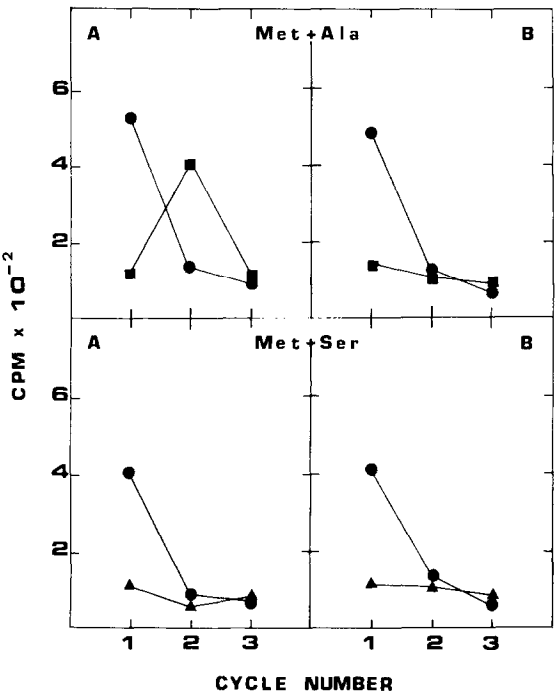

Fig.3 - Partial amino-terminal sequence analysis of the translation products immunoprecipitated by anti-cAAT (*A*) of anti-mAAT (*B*) antibodies. Polyadenylated mRNA was translated using the rabbit reticulocyte lysate system containing (^{35}S)methionine and (^{3}H)alanine (Met + Ala) or (^{35}S)methionine and (^{3}H)serine (Met + Ser). The translation products after immunoprecipitation with the specific antibodies were subjected to 3 cycles of manual Edman degradation. The recovered thiazolinones were dried and their radioactivity measured directly in a liquid scintillation counter set up for double label counting. Diagrams report radioactivity measured after each cycle: ● (^{35}S)methionine; ■ (^{3}H)alanine; ▲ (^{3}H)serine.

(p-mAAT) appeared to have a molecular weight greater than that of mAAT by about 2500 ± 400.

p-cAAT and p-mAAT were synthesized in the presence of both (^{35}S)methionine and (^{3}H)alanine or of both (^{35}S)methionine and (^{3}H)serine and submitted to three Edman degradation cycles; alanine and serine are known to be the amino terminal residues for cAAT and mAAT respectively (8-11). Results, reported in Fig. 3, suggest that: i) methionine is the N-terminal residue of both precursors; ii) alanine is the second residue of p-cAAT; iii) the second residue of p-mAAT is not serine.

These data suggest that the p-cAAT possesses only an extra N-terminal methionine in agreement with the molecular size estimated by SDS - gel electrophoresis; the alanine residue found in the second position could be

that at the N-terminus of the mature protein. On the other hand, the absence of serine either from the second and third positions of p-mAAT suggests in this case the existence of an extra N-terminal peptide sequence, estimated by about 2500 daltons in size from the results of SDS-gel electrophoresis, starting with a methionine residue.

Present results complement those already reported for higher molecular weight precursors for mAAT occurring in chicken heart, $\Delta MW \cong 2000$ (12) and in rat liver, $\Delta MW \cong 3000$ (13). The preliminary sequence data suggest that the extra peptide moiety is located at the N-terminal position.

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