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IMPORT OF A PUTATIVE PRECURSOR OF RAT LIVER MITOCHONDRIAL GLUTAMIC OXALOACETIC TRANSAMINASE INTO MITOCHONDRIA

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SUMMARY

A putative precursor of rat liver mitochondrial glutamic oxaloacetic transaminase which was about 2,000 daltons larger than the subunits of the mature enzyme synthesized *in vitro* was sensitive to proteases (trypsin and chymotrypsin). When this precursor was incubated with isolated mitochondria in the absence of protein synthesis, it was processed to the mature form ; the mature form co-sedimented with mitochondria and was resistant to externally added proteases. Mature enzyme did not compete with this transport.

Glutamic oxaloacetic transaminase (GOT) exists as two different enzymes in higher organisms (1), one located in the cytoplasm (sGOT) (2) and the other in the mitochondria (mGOT; matrix and inner mambrane) (3). Both isozymes are coded by nuclear genomes and are synthesized on cytosolic ribosomes (4). Hence, mGOT must be transferred across both the outer and inner membranes of mitochondria to reach its final location in the mitochondria.

We (5) and Sonderegger *et al.* (6) reported that mGOT was synthesized *in vitro* as a precursor (p-mGOT) and that the precursors of mGOT from rat liver and chicken heart were about 2,000 and 3,000 daltons larger, respectively, than the mature subunits.

We further showed that p-mGOT was processed to its mature form by a mitochondrial membrane preparation from rat liver (5).

Abbreviations used : GOT, glutamic oxaloacetic transaminase [L-aspartate : 2-oxoglutarate aminotransferase (EC 2.6.1.1.)]; sGOT and mGOT, cytoplasmic and mitochondrial isozyme, respectively ; p-mGOT, a putative precursor of mGOT; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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In this paper we report that in association with processing of p-mGOT to its mature form p-mGOT is tranlocated into the mitochondria.

MATERIALS AND METHODS

In Vitro Protein Synthesis

Membrane-free polysomes from rat liver were prepared by the method of Blobel and Potter (7). Polysomes were suspended in 50 mM Tris-HCl, pH 7.5, 25 mM KCl, 5 mM $MgCl_2$ and 20 % (w/v) glycerol (200 A_{260} units/ml).

In Vitro protein synthesis was carried out by the method of Mori *et al.* (8). The standard incubation mixture in a final volume of 50 µl consisted of 28 mM sodium Hepes, 80 mM KCl, 40 mM potassium acetate, 1.6 mM magnesium acetate, 1.1 mM ATP, 28 µM GTP, 9 mM creatine phosphate, 4.4 µg/ml of creatine phosphokinase, 0.3 mM spermidine, 2.2 mM dithiothreitol, a mixture of 28 µM each of all amino acids except methionine, 200 µCi/ml of [^{35}S]-methionine (935 Ci/m mol, New England Nuclear), 400 µl/ml of nuclease-treated reticulocyte lysate (9), and 10-20 A_{260} units/ml of membrane-free polysomes. Incubations were carried out at 30°C for 60 min.

Immunoprecipitation

Immunoprecipitation was performed using antiserum to rat liver mGOT or sGOT and protein A-coated *Staphylococcus* cells (Sigma Chemical Co.) as described previously (5).

Immunoprecipitated prducts were analyzed by SDS-polyacrylamide (10 %) gel electrophoresis (SDS-PAGE) (10) and fluorography (11).

Incubation of p-mGOT with Isolated Mitochondria

Rat liver mitochondria were isolated by the method of Greenwalt (12). After *in vitro* protein synthesis, the mixture of products was centrifuged at 105,000 x g for 60 min at 0°C, and then, the supernatant was incubated with freshly isolated mitochondria (400 µg of protein) in medium consisting of 70 mM sucrose, 220 mM D-mannitol, 2 mM potassium Hepes, 1 mM dithiothreitol and 0.5 mg/ml of bovine serum albumin, pH 7.4, (ISOLATION BUFFER) in the presence or absence of mature mGOT. Incubations were carried out for 0, 30 and 60 min at 30°C.

The mixtures were then centrifuged at 7,000 x g for 15 min at 0°C and the supernatants were analyzed by immunoprecipitation, SDS-PAGE and fluorography.

Precipitates were suspended in the ISOLATION BUFFER, and 250 µg per ml each of trypsin and chymotrypsin were added to the suspension. The mixtures were centrifuged (7,000 x g for 15 min at 0°C), and the precipitates were suspended in the ISOLATION BUFFER, sonicated at 2 kHz for 1.5 min and centrifuged at 105,000 g for 60 min at 0°C. The resulting supernatant fluids (mitochondrial soluble fraction) were analyzed by immunoprecipitation, SDS-PAGE and fluorography.

Miscellaneous

Purification of rat liver GOT isozymes, preparation of antisera against rat liver GOT isozymes and preparation of tritium labelled GOT isozymes were performed as described previously (13) (5). Protein was determined by the method of Lowry et al. (14) with bovine serum albumin as a standard.

Chemicals

All other chemicals used were standard commercial products.

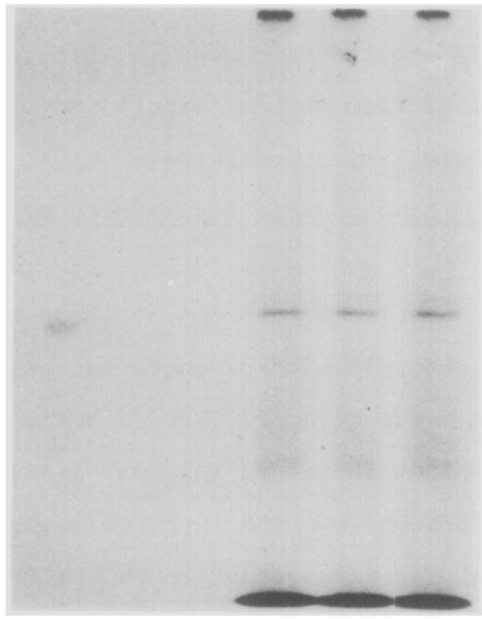

Fig. 1 Proteolytic degradation of p-mGOT synthesized *in vitro*. *In vitro* protein synthesis was performed as described in the MATERIALS AND METHODS. Fifty µl of translated mixture was immunoprecipitated with anti-mGOT antiserum immediately (lane 6) or after standing for 60 min at 4°C (lane 5) or for 30 min at 30°C (lane 4) or after incubation with trypsin and chymotrypsin (250 µg of each/ml) for 60 min at 4°C (lane 3) or for 30 min at 30°C (lane 2). The immunoprecipitated products were analyzed by SDS-PAGE and fluorography. Lane 1 shows tritiated mature mGOT.

RESULTS AND DISCUSSION

Proteolytic Degradation of p-mGOT

One method for demonstrating import of proteins into mitochondria is to incubate them with mitochondria and then measure their resistance to externally added proteases (15). Mature (native) mGOT is resistant to protease digestion, judging from the maintenace of its enzyme activity. We examined whether p-mGOT was accessible to proteases (trypsin and chymotrypsin). As shown in Fig. 1, after tryptic and chymotryptic digestion, p-mGOT was no longer precipitated with its specific antibody. On the basis of the fact

that p-mGOT is susceptible to proteases, it is possible to study the import of p-mGOT into the mitochondria. The conformation of p-mGOT seemed to differ from that of mature mGOT judging from its difference in susceptibility to proteases.

Import of p-mGOT into Mitochondria accompanied by Processing to its Mature Form

Intact mitochondria isolated from rat liver were incubated for 0, 30 and 60 min at 30°C with products synthesized *in vitro*. The mixture was then centrifuged at 7,000 x g for 15 min at 0°C. The resulting supernatant contained residual products synthesized *in vitro* that had not been imported into the mitochondra. The pellet (mitochondria) was resuspended in ISOLATION BUFFER and incubated with trypsin and chymotrypsin. Protease digestion was carried out for 60 min at 4°C to avoid destruction of the mitochondria as far as possible. After protease treatment, mitochondria was recovered by centrifugation and suspended in ISOLATION BUFFER. The suspension was sonicated at 2.0 kHz for 1.5 min to separate the soluble and membrane fractions of mitochondria. The soluble fraction and the supernanant, containing residual products synthesized *in vitro* that had not been imported into the mitochondria, were analyzed by immunoprecipitation, SDS-PAGE and fluorography as described in the MATERIALS AND METHODS.

Fig. 2 shows the patterns on SDS-PAGE of stained protein(A) and fluorography (B). As shown in Fig 2, A (lanes 5-7), endogenous mGOT was recovered in the soluble fraction of mitochondria. There was little leak of mGOT from the mitochondria during incubation as judged by the very small amount of mGOT detected in the supernatant after incubation of mitochondria with *in vitro* products (Fig 2A, lanes 2-4).

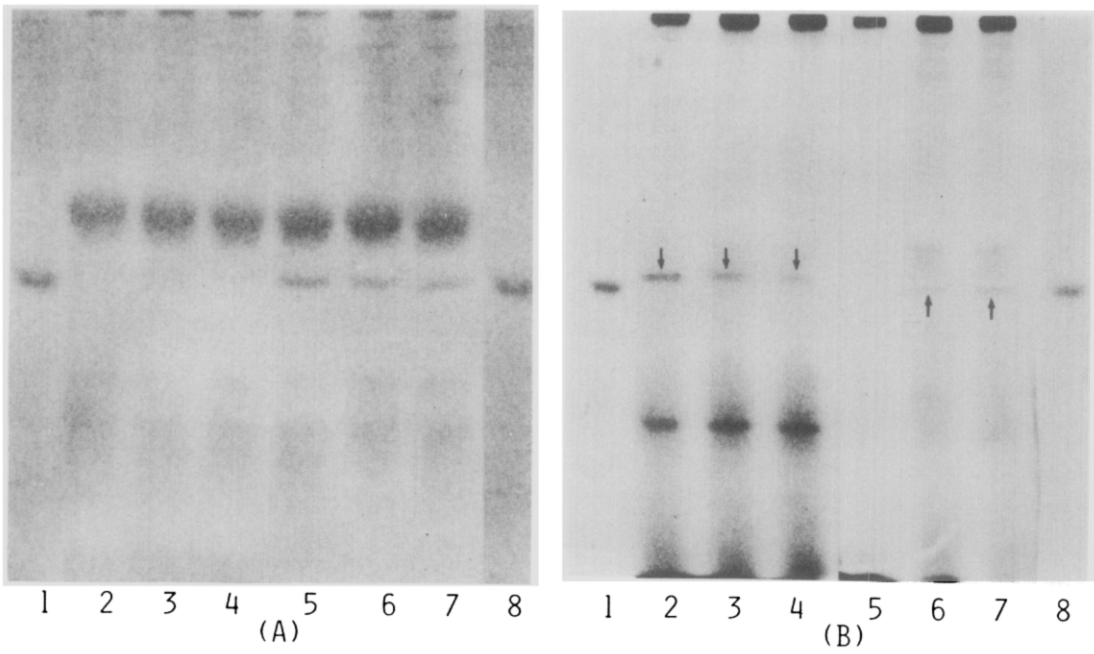

Fig. 2 Import of p-mGOT into mitochondria.
In vitro protein synthesis was performed as described in the MATERIALS AND METHODS. Fifty µl of translated mixture was incubated with isolated mitochondria (400 µg of protein) from rat liver for 0 min (lanes 2 and 5), 30 min (lanes 3 and 6) and 60 min (lanes 4 and 7) at 30°C in the ISOLATION BUFFER. The mixture was centrifuged to recover the mitochondria and the resulting supernatant was analyzed by immunoprecipitation and SDS-PAGE (lanes 2-4). The mitochondrial pellet was resuspended in the ISOLATION BUFFER. The soluble fraction of mitochondria was obtained by sonication after treatment with trypsin and chymotrypsin (250 µg of each/ml) for 60 min at 4°C as described in the MATERIALS AND METHODS. This fraction was subjected to immunoprecipitation and analyzed by SDS-PAGE (lanes 5-7). Lanes 1 and 8, tritiated mature mGOT. (A) and (B) shows the patterns on SDS-PAGE of stained protein and fluorography, respectively. Downward and upward arrows indicate p-mGOT and mature mGOT labelled with [^{35}S]-Met, respectively. In (A), the stained bands above and below the mGOT band are those of heavy and light chains of immunoglobulin, respectively.

After incubation of mitochondria with *in vitro* products, p-mGOT was detected only in the supernatant by SDS-PAGE gel with fluorography (Fig 2B, lanes 2-4). The intensity of the p-mGOT band decreased with time of incubation with mitochondria. On the other hand, the soluble fraction of mitochondria gave a band of only mature mGOT labelled with [^{35}S]-Met which increased with time

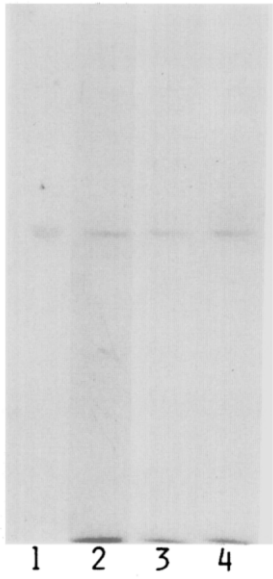

Fig. 3 Absence of inhibition of import of p-mGOT into mitochondria by its mature form.
In vitro protein synthesis was performed as described in the MATERIALS AND METHODS. Fifty µl of translated mixture was incubated for 60 min at 30°C with isolated mitochondria (400 µg of protein) from rat liver in the absence (lane 2) or presence of 5 µg or 20 µg of mature mGOT purified from rat liver (lanes 3 and 4, respectively). The mitochondria were recovered by centrifugation and then incubated with trypsin and chymotrypsin (250 µg of each/ml) for 60 min at 4°C. The mitochondrial soluble fraction was obtained as described in the MATERIALS AND METHODS, subjected to immunoprecipitation and then analyzed by SDS-PAGE and fluorography (lanes 2-4). Lane 1, tritiated mature m-GOT.

of incubation with mitochondria (Fig 2B, lanes 5-7). This mature mGOT appeared to be resistant to proteolysis. As mentioned above, p-mGOT was translocated into mitochondria in association with its processing to the mature form.

On immunoprecipitation with anti-sGOT antiserum, [^{35}S]-Met labelled sGOT was recovered only in the supernatant containing residual products synthesized *in vitro* that were not imported into the mitochondria, and was not found in the soluble fraction of mitochondria (data not shown). In other words, sGOT was not taken up by the mitochondria.

Most mitochondrial proteins are synthesized outside mitochondria as precursors that are usually larger than their mature proteins found in mitochondria. These precursors are imported into mitochondria post-translationally (15-24) dependent on high-energy phosphate bonds inside the mitochondria (21).

Marra *et al.* reported that when rat liver mitochondria were incubated with mature mGOT purified from the same source, it was transported into the mitochondria (25-28).

However, in our experiments, mature mGOT did not compete with import of p-mGOT (Fig 3). Our results suggested that p-mGOT was transported into mitochondria much more readily than its mature form. Further studies are necessary on the pre-piece in p-mGOT to elucidate the mechanism of intracellular translocation of mGOT.

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