In Vitro Synthesis and Processing of a Precursor to Ornithine Aminotransferase*

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Poly(A)* RNA was isolated from liver-free polysomes of rats maintained on a 60% casein diet by sodium dodecyl sulfate-phenol-chloroform extraction and oligo(dT)-cellulose chromatography. Poly(A)* RNA translated in a rabbit reticulocyte lysate system produced a polypeptide of 49,000 daltons that was immunoprecipitated by monospecific, affinity-purified IgG antibodies to ornithine aminotransferase (ornithine-oxo acid aminotransferase, EC 2.6.1.13). This polypeptide is 6,000 daltons larger than mature ornithine aminotransferase when electrophoresed on sodium dodecyl sulfate polyacrylamide gels. One-dimensional peptide mapping demonstrated that this 49,000-dalton polypeptide is structurally related to ornithine aminotransferase. Furthermore, it can be processed to a polypeptide of 43,000 daltons by a rat liver mitochondrial fraction. We have concluded that this polypeptide is a precursor to ornithine aminotransferase.

Ornithine aminotransferase is a mitochondrial matrix enzyme (1) present in rat liver and kidney (2). In these tissues, the synthesis and degradation of ornithine aminotransferase are subject to several hormonal and dietary controls (3–5). As part of a program designed to investigate the molecular mechanisms of ornithine aminotransferase induction, we have studied the translational product of cytoplasmic polysomes synthesizing this enzyme. Other mitochondrial matrix enzymes that are synthesized on cytoplasmic polysomes have been shown to be synthesized as larger precursor molecules (6–12).

In this communication, we report that ornithine aminotransferase is synthesized as larger precursor molecules (6–12).

**MATERIALS AND METHODS**

**Enzyme Purification and Preparation of Antiornithine Aminotransferase γ-Globulin—**Ornithine aminotransferase was purified to homogeneity by the procedure of Peraino et al. (13). A single band was detected by SDS-polyacrylamide gel electrophoresis. Rabbit anti-ornithine aminotransferase γ-globulin was prepared as described previously (2). This antibody was monospecific as determined by Ouchterlony double diffusion analysis (2). The antibody preparation was purified further by affinity chromatography. 30 mg of purified ornithine aminotransferase was coupled to CNBr-activated Sepharose 4B as described by the manufacturer (Pharmacia Fine Chemicals, Uppsala, Sweden).

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‡The abbreviation used is: SDS, sodium dodecyl sulfate.

Piscataway, NJ and affinity chromatography was conducted by the method of Gough and Adams (14).

**Isolation of Poly(A)* RNA from Rat Liver—**Male Sprague-Dawley rats (200 to 300 g) were maintained on a 60% casein diet for at least 3 days before killing in an attempt to induce ornithine aminotransferase mRNA (2). Free polysomes were prepared and poly(A)* RNA was isolated by SDS-phenol-chloroform extraction and oligo(dT)-cellulose chromatography as described previously (15).

**In Vitro Translation of Precursor to Ornithine Aminotransferase γ-Globulin—**Poly(A)* RNA was translated in a nucleoside-treatment rabbit reticulocyte lysate system (16) as described by Gonzalez and Kasper (17). K+ and Mg2+ concentrations were 100 mM and 0.75 mM. Decacylated rat liver tRNA was added at 100 μg/ml and [35S]methionine at 500 μCi/ml. Translation was allowed to proceed for 45 min at 30 °C and quenched by cooling on ice. To determine total counts incorporated into protein, 2-ml aliquots of the reaction mixture were spotted onto Whatman No. 1 filter paper. The filter paper was boiled in 10% trichloroacetic acid for 10 min, then washed twice each in 5% trichloroacetic acid, methanol, and ether. The filters were counted in 10 ml of OCS scintillator mixture (New England Nuclear).

An equal volume of TNM buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM MgCl2) was added to the remainder of the lysate mixture which was then centrifuged at 25,000 rpm for 2 h in a Beckman type 25 rotor. The resulting supernatant was adjusted to 1% sodium deoxycholate, 1% Triton X-100, and 10 mM methionine, 10 μg of purified ornithine aminotransferase, and 100 μg of anti-ornithine aminotransferase γ-globulin were added and the mixture was incubated at 25 °C for 1 h, then at 4 °C overnight. Immunoprecipitates were washed by pelleting through 3-ml 1 M sucrose cushions containing 0.15 M NaCl, 10 mM Tris-Cl, pH 7.6, 10 mM methionine, 1% sodium deoxycholate, and 1% Triton X-100 by centrifuging in a Beckman SW 60Ti rotor at 20,000 rpm for 30 min. Pellets were transferred to 1.5-ml microfuge tubes and washed several more times by suspension in the above cushion buffer lacking sucrose and pelleting in a microfuge.

Immunoprecipitates were analyzed on 10% polyacrylamide-SDS slab gels using the system of Laemmli (18). Gels were stained in Coomassie blue to visualize the position of mature ornithine aminotransferase, treated with ENHANCE (New England Nuclear), dried, and fluorographed by exposing Kodak XAR-5 film at −70 °C.

In Vitro Processing of Pre-ornithine Aminotransferase by a Mitochondrial Fraction—A rat liver mitochondrial fraction was isolated essentially as described (14, 19), except that the isolation buffer consisted of 0.25 M sucrose, 20 mM Tris-Cl, pH 7.6, 1 mM ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid. Processing was conducted as described by Conboy and Rosenberg (33). One volume of mitochondria suspended in isolated buffer was added to 2 volumes of translation products and the mixture was incubated at 27 °C for 1 h. Mitochondria were pelleted by spinning the mixture for 2 min at 13,000 × g in an Eppendorf microfuge. The pellet and supernatant fractions were subjected to immunoprecipitation with anti-ornithine aminotransferase γ-globulin and the immunoprecipitates were analyzed on 10% polyacrylamide-SDS slab gels as described above.

**RESULTS**

Ornithine aminotransferase exhibits a molecular weight of 43,000 on SDS-polyacrylamide gels (2). To determine whether this enzyme, like several other mitochondrial matrix enzymes...
(4-10), is synthesized as a precursor polypeptide, we translated poly(A) + RNA from rat liver-free polysomes in a rabbit reticulocyte lysate system. The resulting products were subjected to immunoprecipitation with monospecific, affinity-purified anti-ornithine aminotransferase IgG, and the immunoprecipitate was analyzed by SDS-polyacrylamide gel electrophoresis. A fluorograph of the dried gel is shown in Fig. 1. The only product observed had a molecular weight of 49,000 (left lane), 6,000 larger than that of the mature enzyme (right lane) (2, 20).

To determine whether this polypeptide is structurally as well as antigenically related to ornithine aminotransferase, we conducted one-dimensional peptide mapping by the procedure of Cleveland et al. (21). Fig. 2 shows that the peptide maps of the 49,000-dalton polypeptide and mature ornithine aminotransferase are very similar, but not identical. This is the result expected if the 49,000-dalton polypeptide is a larger precursor to mature ornithine aminotransferase. Therefore, we refer to this polypeptide as pre-ornithine aminotransferase.

To obtain further evidence that the 49,000-dalton polypeptide is a genuine precursor to ornithine aminotransferase, we tested whether it could be specifically processed to a polypeptide of 43,000 daltons by a mitochondrial fraction. Fig. 3 demonstrates that such processing does occur. Control experiments (not shown) demonstrated that incubation in the presence of equivalent quantities of rat liver cell sap or rough and smooth membrane protein did not result in any change in the 49,000-dalton polypeptide. The experiment shown in Fig. 3 demonstrates that the extent of processing is dependent on the quantity of mitochondrial protein added, and that the resulting ornithine aminotransferase is associated with the mitochondria. Aliquots of [35S]methionine-labeled translation products were incubated with increasing quantities of mitochondrial protein. Mitochondria were pelleted and the resulting supernatants and pellets were subjected to immunoprecipitation with anti-ornithine aminotransferase γ-globulin and the immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis. Mature ornithine aminotransferase was associated exclusively with the pelleted (mitochondrial) fractions (lanes e to g), whereas the unprocessed precursor was found predominantly in the supernatant fractions (lanes a to d).

### DISCUSSION

The following observations indicate that ornithine aminotransferase is synthesized as a larger precursor polypeptide: 1) The in vitro translation product immunoprecipitated by affinity-purified, monospecific anti-ornithine aminotransferase IgG is 6,000 daltons larger than the mature enzyme on SDS-
polyacylamide gels. 2) The in vitro translation product exhibits a similar, but not identical one-dimensional peptide map to ornithine aminotransferase. 3) The 49,000-dalton in vitro translation product is processed in the presence of a mitochondrial fraction to a polypeptide of 43,000 daltons, identical in size with mature ornithine aminotransferase, and the processed polypeptide co-sediments with mitochondria.

Many (6–12, 22–27) but not all (28–31) mitochondrial proteins that are synthesized on cytoplasmic polysomes are produced as soluble precursor proteins 2,000 to 6,000 daltons larger than the mature proteins. Current evidence suggests that the conversion of these precursors to their mature intramitochondrial forms is a post-translational event. This view is supported by both in vitro pulse-labeling experiments (8, 22, 24–27) as well as in vitro experiments employing purified mitochondria (9, 22, 24, 32, 33). Despite this common denominator, there is likely to exist some heterogeneity in the mechanisms of transport and integration of proteins into the various mitochondrial compartments. This is suggested by the observations that not all mitochondrial proteins are synthesized as larger precursor forms (25–28), and that the individual precursor forms may differ considerably in length. This latter observation holds true for mitochondrial proteins of the same compartment. For example, the enzymes carbamyl phosphate synthetase I, ornithine transcarbamoylase, aspartate aminotransferase, f-aminolevulinic acid synthetase, glutamic oxaloacetic transaminase, and ornithine aminotransferase are synthesized as precursor molecules that are 5,000 to 5,500 (7, 9), 3,400 to 4,000 (6, 32), 3,000 (12), 6,000 (10), 2,000 (11), and 6,000 daltons (this report), respectively, larger than their forms found in the mitochondrial. Some heterogeneity may also exist in the subcytoplasmic location of polysomes synthesizing specific mitochondrial proteins. Rat liver carbamyl phosphate synthetase is synthesized on membrane-free polysomes (8), whereas three subunits of F,-ATPase of yeast are synthesized preferentially on polysomes bound to mitochondria (34).

We conclude that ornithine aminotransferase is synthesized as a precursor polypeptide of 49,000 daltons, 6,000 daltons larger than the mature enzyme. In this respect, it is similar to several other mitochondrial matrix enzymes that have been examined (6–12). The details of the mechanism(s) of transport of polypeptides across both mitochondrial membranes into the matrix are still largely unknown. We have recently succeeded in cloning DNA complementary to ornithine aminotransferase mRNA (35). Ornithine aminotransferase can be induced and maintained in long term primary hepatocyte cultures (36). Thus, this enzyme offers an excellent prospect for elucidating these mechanisms.

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Addendum—While this manuscript was under review, a communication appeared by Hayashi et al. (1981) J. Biochem. (Tokyo) 90, 1229–1232 purporting to demonstrate that ornithine aminotransferase is not synthesized as a precursor polypeptide. We have observed that protease activity present in rabbit liver fractions and/or the liver mRNA-programmed reticulocyte lysate itself will process pre-ornithine aminotransferase to the mature enzyme. When incubated at 37 °C rather than 27 °C, this processing activity is considerably enhanced. Under the conditions of immunoprecipitation and in vitro processing by mitochondria described in this paper, this activity is negligible. This observation may account for the different results obtained by Hayashi et al. and us.

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