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Evidence for intra-mitochondrial degradation of the extrapeptide of ornithine aminotransferase

Wei-Ping Ren, Hideyu Ono, and Syozo Tuboi

Department of Biochemistry, Yamagata University School of Medicine, Yamagata 990-23, JAPAN

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When rat liver mitochondria that had imported a synthetic extrapeptide of ornithine aminotransferase (composed of 34 amino acids) were incubated at 25 °C, the extrapeptide in their matrix was degraded inside the mitochondria. The degradation of the extrapeptide did not depend on energy either inside or outside the mitochondria. The degrading activity was found exclusively in the mitochondrial soluble fraction and only inhibited by N-ethylmaleimide of eight protease-inhibitors tested. These observations show that the extrapeptide cleaved from the precursor of the mitochondrial protein in the mitochondria is degraded by some ATP-independent proteases inside the mitochondria.

The precursors of most mitochondrial proteins imported into mitochondria have been shown to be converted to their mature forms by cleavage of their extrapeptide by a processing peptidase in the mitochondrial matrix (1-8). The extrapeptide cleaved from the precursor has been assumed to be degraded to amino acids by some intra-mitochondrial proteases, but as yet there has been no direct evidence for the intra- mitochondrial degradation of this extrapeptide.

Previously we reported (9) that the extrapeptide of ornithine aminotransferase (OAT; EC 2.6.1.13), composed of 34 amino acids, was efficiently imported into the mitochondrial matrix *in vitro*, and its import was depending on the mitochondrial membrane potential ($\Delta\psi$). The extrapeptide imported into the matrix was supposed to be degraded by some proteases in the mitochondria. In this communication we report evidence for the intra-mitochondrial degradation of the extrapeptide of OAT and show that the degradation process does not require any energy such as that of ATP.

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MATERIALS AND METHODS

Preparation of mitochondria and their soluble and membrane fractions: Mitochondria were prepared as described previously (10) from the liver of male Wistar rats of about 200 g. For subfractionation of mitochondria rats were injected with Triton WR 1339 at the dosage of 85 mg per 100 g body weight 84 h before sacrifice as described by Leighton et al. (11). The mitochondria prepared from liver of these rats by the same way as described above were further purified with a discontinuous sucrose density gradient centrifugation as described by Yamamoto et al. (12). The purified mitochondria were used for preparation of the mitochondrial membrane and matrix fractions. The mitochondia purified were suspended in 5 mM potassium phosphate buffer (pH 7.5) and sonicated. The sonicates were centrifuged at 320,000 x g for 30 min. The supernatant was used as the mitochondrial soluble fraction. The precipitates (the mitochondrial membrane fraction) were washed with 10 mM Hepes KOH buffer (pH 7.6) containing 500 mM potassium acetate. The washed precipitates were suspended in 5 mM potassium phosphate buffer (pH 7.5) and used as the mitochondrial membrane fraction.

Import of ^{3}H-labeled extrapeptide of OAT into mitochondria: The extrapeptide of OAT was synthesized chemically by Nikkaki Co., Ltd. (Tokyo) and labeled with N-succinimidyl [2,3-^{3}H] propionate by the method of Bolton and Hunter (13) as described in our previous paper (9). This synthetic peptide is refered to as "the extrapeptide" in this text. Mitochondria with imported ^{3}H-labeled extrapeptide were prepared by incubating them with the labeled peptide in the presence of energy-generating system such as succinate and a rabbit reticulocyte lysate (9) at 25 °C for 3 min. The mitochondria reisolated and washed were used for experiment of the degradation of the peptide.

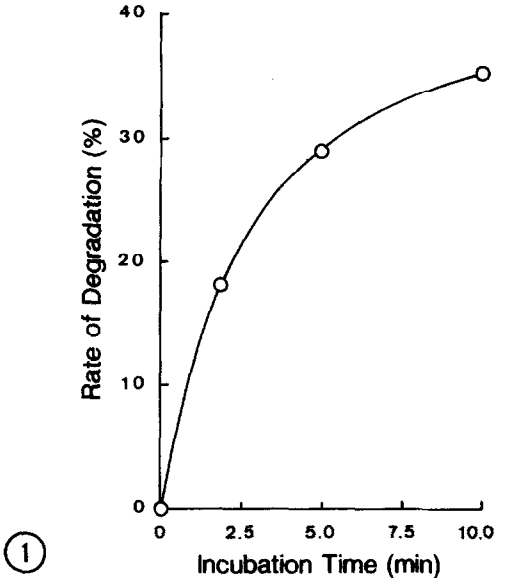
Assay of degradation of the peptide: When the solution was acidified with 10% TCA, the extrapeptide composed of 34 amino acids was almost stoichiometrically recovered in the precipitate with bovine serum albumin as a carrier. Thus degradation products could be separated from the intact extrapeptide by treatment with TCA and centrifugation. In experiments mitochondria with imported ^{3}H-labeled extrapeptide were reisolated by centrifugation at 10,000 xg for 5 min and washed twice at 0°C with washing buffer (pH 7.6) composed of 10 mM Hepes-KOH, 0.48 M mannitol, 2 mM magnesium acetate, 150 mM potassium acetate and 0.5 mg/ml bovin serum albumin. They were then resuspended in 0.5 ml of the washing buffer and reincubated at 25°C. The reaction was terminated by addition of 1.5 mg of bovine serum albumin as a carrier and 0.5 ml of 20% TCA, and the mixture was centrifuged. The amount of extrapeptide converted to TCA-soluble materials was then determined by measuring the radioactivity of the TCA-soluble fraction.

RESULTS AND DISCUSSION

Intra-mitochondrial degradation of the extrapeptide: In preliminary experiments we found that a lysate of rabbit reticulocytes contained a protease(s) which actively degraded the extrapeptide and was not inhibited by leupeptin or pepstatin. Therefore, assay of intra-mitochondrial degradation of the extrapeptide had to be carried out in the absence of a reticulocyte lysate.

Therefore, we used reisolated mitochondria that had imported ^{3}H-labeled extrapeptide into their matrix. When the isolated mitochondria containing ^{3}H-labeled extrapeptide were incubated in the absence of rabbit reticulocyte lysate at 25°C, the extrapeptide was degraded time-dependently, and about 35% of the amount present initially in the mitochondria was converted to TCA-soluble materials in 10 min, as shown in Fig. 1. This degradation was not affected by addition of an ATP-generating system to the reaction mixture, indicating that it did not require extra-mitochondrial ATP.

Energy-independent breakdown of the extrapeptide: The breakdowns of some intra-cellular proteins require an energy source such as ATP (14-17) and ATP-dependent proteolysis has also been observed in mitochondria (18, 19). Thus, conceivably, the intra-mitochondrial degradation of the extrapeptide might be an



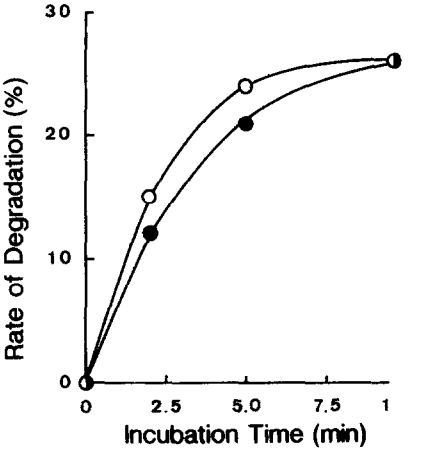


Fig. 1. Time course of degradation of the extrapeptide. Mitochondria (1.5 mg) containing ^{3}H-labeled extrapeptide (690 cpm as TCA-insoluble fraction, about 1.3 ng of the extrapeptide) were incubated at 25°C for the times indicated in the figure. The rate of degradation of the extrapeptide is expressed as the radioactivity in the TCA-soluble fraction as a percentage of that of the initial TCA-insoluble fraction.

Fig. 2. Effect of addition of excess unlabeled extrapeptide to the reaction mixture. Mitochondria containing ^{3}H-labeled extrapeptide (1150 cpm as TCA-insoluble fraction) were incubated in the absence (o) or presence (•) of the cold extrapeptide (2.5 μg, representing over 100 times to the amount of ^{3}H-labeled extrapeptide). Other experimental conditions were as for Fig. 1.

energy-dependent process. To exclude this possibility, we incubated isolated mitochondria containing ^{3}H-labeled extrapeptide with apyrase (4 units/ml) for 10 min at 25°C to decrease the intra-mitochondrial ATP concentration. Results showed that this treatment with apyrase did not affect degradation of the extrapeptide. Addition of 50 μM carbonyl cyanide m-chlorophenylhydrazone (CCCP), which is an uncoupler of the electron transport system, also had no effect on intra-mitochondrial degradation of this peptide. These observations suggest that the intra-mitochondrial degradation of the extrapeptide does not require either ATP or another energy source; that is, it is not due to ATP-dependent proteolysis.

Other evidence for intra-mitochondrial degradation of the extrapeptide: As mitochondrial preparations usually contain a considerable amount of lysosomes, which participate in the digestions of various peptides, the extrapeptide imported into mitochondria might be degraded after its export from the mitochondria. To exclude this possibility, mitochondria containing ^{3}H-labeled extrapeptide were incubated in the presence of excess unlabeled extrapeptide. As shown in Fig. 2, degradation of the ^{3}H-labeled extrapeptide was not inhibited by the presence of excess unlabeled extrapeptide from the beginning of the reaction, indicating that the degradation of this peptide occurs inside, not outside the mitochondria. This means that lysosomes, which may contaminate the mitochondrial fraction, are not responsible for its degradation.

Degradation of extrapeptide by the mitochondrial soluble fraction: When the extrapeptide (30,000 cpm) was incubated with 200 μg of the mitochondrial soluble fraction at 25°C for 2.5 min at pH 7.5, about 56% of the extrapeptide was degraded. But the mitochondrial membrane fraction (200 μg) did not degrade the extrapeptide. The activity at pH 5.5 was only 30% of that at pH 7.5, indicating that this protease is a neutral protease located in the mitochondria and distinct from lysosomal proteases. Phenylmethylsulfonylfluoride(PMSF), EDTA, EGTA, leupeptin, pepstatin, chymostatin, and aprotinin did not inhibit the degradation. About 50% inhibition was observed by pre-treatment of the mitochondrial soluble fraction with N-ethylmaleimide(NEM) at the final concentration of 20 mM. This inhibitory effect was canceled out when NEM was treated with dithiothreitol before use. The intra-mitochondrial location of

this protease seems to be the matrix, because the protease was not released from mitochondria with mild osmotic shock which easily released sulfite oxidase, an enzyme localized in the inter-membrane space.

The present results show that the extrapeptide cleaved from the precursor of the mitochondrial protein in the mitochondria is degraded by one of thiol protease located in the mitochondrial matrix, and that this degradation does not require any energy source such as ATP.

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