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EXIT OF PROTEINS AND FRAGMENTS THEREOF FROM MITOCHONDRIA IS ACCELERATED BY THE IMPORT OF CYTOSOLIC SYNTHESIZED PROTEINS

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SUMMARY Most mitochondrial proteins are synthesized on cytosolic ribosomes and imported into mitochondria. Incubation of ^{35}S-methionine labeled mitochondria from rat hepatocytes with proteins synthesized in a cell-free system, using messenger RNA from rat liver, dramatically increased the release of mitochondrial proteins and fragments thereof into the medium. Since the synthesized proteins include cytosolic precursors of mitochondrial proteins, our results strongly suggest that import of proteins from the cytosol into mitochondria influences the half-life of proteins in these organelles. The use of this simple approach — i.e. combining the study of protein import and exit with mitochondria — to further clarify intracellular protein turnover and its regulation is suggested.

Although much is known regarding protein synthesis, including the fact that many proteins, particularly mitochondrial are synthesized in the form of larger precursors (1,2), little is known regarding the mechanism of intracellular protein degradation, and particularly its regulation. Under steady state conditions subcellular components, including proteins, are broken down and resynthesized many times during cellular life span (3,4).

The interaction between degradation and synthetic processes which maintain the intracellular proteins essentially constant have been investigated using different approaches including liver regeneration, use of protein synthesis inhibitors or inductors, etc. The strongest evidence thus far available sug-

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gesting a close link between synthesis and breakdown of proteins is that inhibitors of protein synthesis increase the stability of some liver enzymes induced to high levels by a number of procedures (see 3 for a review). In this paper we present evidence using a simple experimental approach for a direct relationship between mitochondrial protein release and the synthesis of mitochondrial protein precursors.

The approach presented here is the in vitro incubation of ^{35}S-methionine-labeled mitochondria from rat hepatocytes with or without the cytosolic precursors of mitochondrial proteins, synthesized in a cell-free system using mRNA from rat liver. We show that the presence of proteins synthesized de novo outside the mitochondria dramatically increases the release of mitochondrial proteins, as judged by the liberation of trichloroacetic acid-precipitable radioactivity derived from mitochondrial proteins into the medium.

MATERIALS AND METHODS

Preparation of hepatocytes.

Hepatocytes were prepared from Wistar rats using collagenase (6). The yield of viable cells was improved by centrifugation of the hepatocyte suspension on a Metrizamide cushion as described by Seglen (7). Viability was always better than 75% after the incubation period, as assessed by trypan blue exclusion.

Labeling of proteins in hepatocytes.

Hepatocytes (2.5×10^7 viable cells/ml) were incubated in a shaking bath at 37°C in methionine-free Eagle's minimum essential medium (modified) with Earle's salts, 2 g/l sodium bicarbonate and freshly supplemented with L-glutamine (292.3 mg/ml). Pulse period (2 h) started by adding 8 µl/ml of ^{35}S-methionine (1390 Ci/mmol).

Isolation of mitochondria from hepatocytes.

After pulse, hepatocytes were spun down for 10 min at 650 g, at 4°C, resuspended in 2 volumes of 70 mM sucrose, 220 mM D-mannitol, 2 mM 4-(2-hydroxethyl)-1-piperazine-ethanesulfonic acid (Hepes), pH 7.4, and then homogenized in a Potter-Elvejhem homogenizer at 1500 rpm, 10 strokes. One volume of the same solution was added and the homogenate was centrifuged for 10 min at 650 g and 4°C. Supernatant was centrifuged at 8,000 g, 15 min, the pellet was washed twice and the resulting mitochondrial fraction was resuspended in the homogenization medium to give 3.5 mg/ml protein.

Cell-free protein synthesis.

The assay mixture (1 ml, pH 7.6) contained 50 mM Hepes, 215 mM potassium acetate, 2.2 mM magnesium acetate, 2 mM ATP, 50 μM GTP, 15 mM creatine phosphate, 540 μg of creatine phosphokinase, 4 mM dithiothreitol, 18 mM each of an aminoacid mixture minus methionine, 8 mM of L-methionine, 50 μg rabbit liver tRNAs, 2 mM each of protease inhibitors (antipain, leupeptin, chymostatin and pepstatin), 1 mg of rat liver mRNA and 40 μl of rabbit reticulocyte lysate. For controls, 10 mM potassium phosphate buffer, pH 7.6, was used instead of rabbit reticulocyte lysate. The reaction mixture was incubated for 90 min at 30°C and then 100 μl 0.2 M L-methionine was added.

Incubation of mitochondria with the cell-free protein synthesis mixture.

Aliquots of 50 μl of mitochondrial suspension were added to tubes containing 500 μl 0.25 M sucrose in 20 mM Hepes, pH 7.2, and 50 μl of the cell-free protein synthesis mixture or eluate from Sephadex G-50 fine gel filtration carried out as in (8). Tubes were shaken after addition of all components and incubated at 30°C in a shaking bath for 0, 30, 60 and 120 min. At the indicated times 100 μl of freshly isolated rat liver mitochondria (60 mg protein/ml) were mixed in (to aid in coprecipitation and thus to minimize blanks) and then centrifuged 10 min in an Eppendorf centrifuge. Sediment was resuspended in 50 μl of 10 mM potassium phosphate buffer containing 0.15 mM each of protease inhibitors (antipain, leupeptin, chymostatin and pepstatin) and 500 μl bidistilled water. Supernatants and resuspended sediments were precipitated with 5% trichloroacetic acid (TCA) containing 5 mg/ml bovine serum albumin. After dissolving the precipitated protein in 1 N NaOH the TCA-soluble and precipitable radioactivities were determined by liquid scintillation counting and expressed as percentage of total radioactivity in both sediments and supernatants.

General procedures.

Isolation of rat liver polysomal RNA was carried out as described previously (9). Rat liver mitochondria were obtained from Wistar rats (10). Carbamyl phosphate synthase and N-acetyl-β-D-glucosaminidase were assayed as in (11). Protein determinations were done by the method of Bradford (12), using bovine serum albumin as standard.

Materials

L-(^{35}S) methionine was purchased from the Radiochemical Centre, Amersham. Protease inhibitors, creatine phosphokinase and rabbit liver tRNAs were obtained from Sigma. Eagle's minimal essential medium was from Flow Labs (Irvine) and collagenase was from Boehringer.

RESULTS AND DISCUSSION

Isolation of mitochondria from rat hepatocytes.

Mitochondria fractions obtained from rat hepatocytes were evaluated morphologically by electron microscopy (their appearance was normal, data not shown), and enzymatically by measuring carbamyl phosphate synthase and N-acetyl-β-D glucosamini-

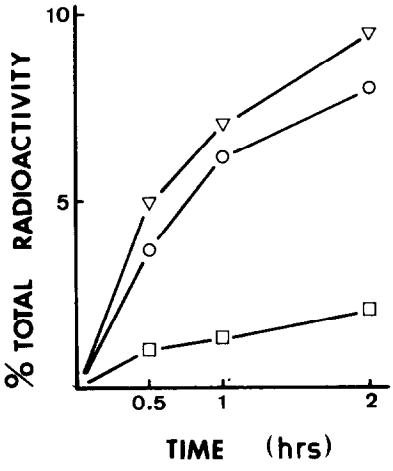

Figure 1. TCA-precipitable radioactivity released by labeled mitochondria during incubation with the products of the cell-free synthesis.
Incubation of labeled mitochondria isolated from rat hepatocytes with the in vitro protein synthesis mixture was performed as described in Materials and Methods. Mitochondria were incubated with: □ cell-free protein synthesis mixture (without rabbit reticulocyte lysate); ○ complete cell-free protein synthesis mixture; ▽ eluate from Sephadex G-50, fine, gel filtration of the complete cell free protein synthesis mixture.

dase activities. Specific activities measured under standard conditions (11) were similar to those obtained with rat liver mitochondrial fractions.

Incubation of pre-labeled mitochondria with the products of the "in vitro" synthesis releases TCA-precipitable radioactivity.

Fig. 1 shows the percentage of TCA-precipitable radioactivity released during the incubation period. As shown, addition of the products of the cell-free synthesis of rat liver proteins yields, after 2 h incubation, a 4-fold increase in the release of TCA-insoluble radioactivity to the medium when compared to similar incubation in which the reticulocytes had been omitted. Since the eluate from the gel filtration after the cell-free synthesis step gives essentially the same values as those observed without filtration, it can be concluded that the observed effect is due to high molecular weight components of

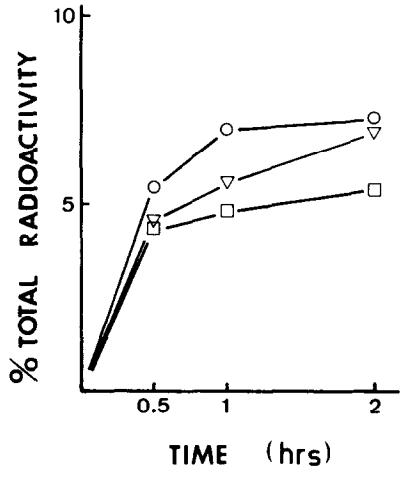

Figure 2. TCA-soluble radioactivity released by labeled mitochondria during incubation with the products of the cell-free protein synthesis.
Incubation of labeled mitochondria isolated from rat hepatocytes with the in vitro protein synthesis mixture was performed as described in Materials and Methods. Mitochondria were incubated with: □ cell-free protein synthesis mixture (without rabbit reticulocyte lysate); ○ complete cell-free protein synthesis mixture; ▽ eluate from Sephadex G-50, fine, gel filtration of the complete cell free protein synthesis mixture.

the cell-free synthesis mixture, including cytosolic precursors of mitochondrial proteins. This indicates that the import of mitochondrial proteins from the cytosol into mitochondria, as seen by fluorography in other experiments (not shown) (13), will be reflected in the apparent half-life of proteins and thus in protein turnover. Fig. 2 shows the percentage of TCA-soluble radioactivity released during the incubation period. The release of TCA-soluble radioactivity was about the same in all cases.

Mitochondrial enzymes have vastly different half-lives ranging from 30 min for δ-aminolevulinate synthase to 7.7 days for carbamyl phosphate synthase (14). Therefore, bulk or unregulated autophagy as the main regulating mechanism of turnover seems unlikely. Mitochondrial proteins are degraded by different mechanisms (15-18), including A) within the mitochondria, B) in the cy-

tosol after exit from the mitochondria, and C) after initiation of degradation in the mitochondria and further degradation in the cytosol. Our results clearly support the last two mechanisms. However, more experiments are necessary to clarify them, particularly the quantitation and specificity of pathways B and C. Thus far there were no methods described which permitted such study; the simple technique described here should be useful for its clarification.

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