An ammonium sulphate fraction from rabbit reticulocytes increases the release of proteins from rat liver mitochondria

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Incubation of [^{35}S]methionine labeled mitochondria from rat liver with rabbit reticulocyte lysate under the same conditions as those used in the import of mitochondrial protein precursors results in the release of mitochondrial proteins to the medium. Fractionation of the lysates with ammonium sulphate yields a fraction, essentially free of haemoglobin, which exhibits higher activity for the release of mitochondrial proteins than the starting lysate. The fraction has a molecular mass of > 10 kDa and is heat-sensitive. The release is insensitive to inhibitors of reticulocyte lipoxygenase.

Protein *Mitochondria* *Rat liver* *Reticulocyte* *Precursor*

1. INTRODUCTION

Under steady-state conditions protein synthesis and protein degradation should be in balance. While much is known regarding mitochondrial protein synthesis, little is known about the regulation and/or the systems involved in degradation of mitochondrial proteins.

Mitochondria acquire the majority of their proteins by importing them from the cytoplasm where many of them are synthesized in the form of larger precursors [1]. In a previous communication [2] we presented evidence in a cell-free system from rabbit reticulocyte lysate for concomitant entry of mitochondrial protein precursors into mitochondria and release of mitochondrial proteins. Here, we show that, in the absence of synthesis of mitochondrial protein precursors, a component(s) from the reticulocyte lysate markedly stimulates the release of proteins from mitochondria to the medium.

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Abbreviation: FRL_{55}, fraction obtained by saturation to 55% with ammonium sulphate of reticulocyte lysates followed by centrifugation

2. MATERIALS AND METHODS

2.1. *Materials*

L-[^{35}S]Methionine (1200 Ci/mmol, 11 mCi/ml) was purchased from the Radiochemical Center, Amersham.

2.2. *Fractionation of rabbit reticulocyte lysates*

Five ml of reticulocyte lysate, prepared from phenylhydrazine-treated rabbits as in [3], were brought to 55% saturation with solid ammonium sulphate at 4°C. Precipitated protein was separated by centrifugation (10 min, 12000 × *g* at 4°C). The precipitate was taken in 2 ml of 25 mM Tris–HCl (pH 7.6). The supernatant and the precipitate were passed through PD-10 columns (Pharmacia) of Sephadex G-25 (medium) which had been equilibrated with 25 mM Tris–HCl (pH 7.6). Both fractions were stored at −80°C.

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2.3. *Cell-free protein synthesis with rat liver extract*

Rat liver extracts were prepared, according to Ogata et al. [4], in medium A1 (50 mM Tris–HCl, 5 mM $MgCl_2$, 25 mM KCl, 25 mM NH_4Cl, 5 mM 2-mercaptoethanol, 0.25 M sucrose, pH 7.6); the protein content was 24 mg/ml. The standard synthesis mixture (30 μl) contained 80 mM KCl, 0.3 mM $MgCl_2$, 10 mM creatine phosphate, 0.6 μM ATP, 0.25 μM GTP, 1 μg creatine kinase, 100 μg/ml chloramphenicol, 20 μl rat liver extract, 4 μl [^{35}S]methionine and a mixture of the remaining 19 amino acids, 50 μM each. Incubations were carried out for 60 min at 30°C.

2.4. *Import of proteins by isolated mitochondria*

Rat liver extract (225 μl) that had synthesized proteins labeled with [^{35}S]methionine was incubated with mitochondria (15.6 mg of mitochondrial protein) in a final volume of 430 μl medium A1 containing 2 mM succinate. Samples were incubated at 30°C for 30 min. Samples were immediately centrifuged at 10000 × *g* for 2 min in an Eppendorf centrifuge. Mitochondria were washed 6 times with 1 ml of medium A1.

2.5. *Incubation of mitochondria with the rabbit reticulocyte lysate and their fractions*

Mitochondria containing imported labeled proteins synthesized by a cell-free rat liver extract, were incubated with the rabbit reticulocyte lysate or fractions thereof in a medium containing 0.3 M sucrose and 25 mM Tris–HCl (pH 7.6) for 20 min at 37°C unless otherwise indicated.

Mitochondria were then centrifuged (10 min, 10000 × *g*) and suspended in 0.5 M Tris–HCl (pH 6.8) plus 2.5% sodium dodecyl sulphate. Supernatants and suspended sediments were brought to 10% trichloroacetic acid by addition of 1.5 vols of 25% trichloroacetic acid and the trichloroacetic acid-precipitable radioactivities were determined [5].

2.6. *Miscellaneous*

Mitochondria were obtained by the method of Kun et al. [6]. Protein was determined by the biuret-deoxycholate method [7]. Amicon's Centricon microconcentrators were used to separate the low and high molecular mass components from reticulocyte lysates.

3. RESULTS AND DISCUSSION

As already shown with mitochondria from hepatocytes [2], incubation of labeled [^{35}S]mitochondria with lysates from rabbit reticulocytes released trichloroacetic acid-precipitable radioactivity. This release increased linearly up to 20 min and then decreased slightly (fig.1), probably due to degradation of the released proteins by proteases in the reticulocytes [8].

The component(s) from the reticulocyte lysate responsible for the release of protein from mitochondria can be precipitated by ammonium sulphate at 55% saturation. Fig.2 shows the stimulatory activity of this fraction on the release of trichloroacetic acid-precipitable radioactivity from mitochondria compared with the complete lysate. The supernatant of the precipitation with ammonium sulphate (containing the bulk of haemoglobin) not only does not stimulate the liberation of radioactivity to the medium but has an inhibitory effect on the activity of the ammonium sulphate fraction.

The activity of the ammonium sulphate fraction is only slightly inhibited by salicylhydroxamic acid, the best known inhibitor of reticulocyte lipoxygenase [9]; at 1 mM it inhibits the action of the

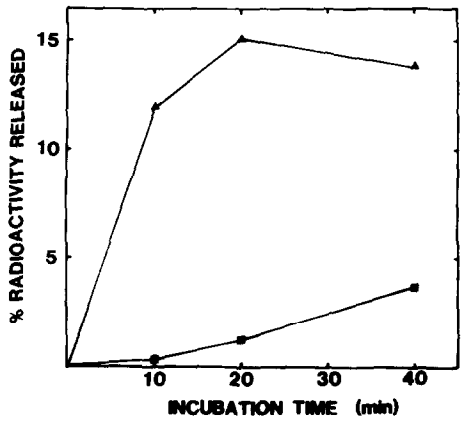

Fig.1. Release of trichloroacetic acid-precipitable radioactivity from mitochondria. Protein, synthesized and labeled with [^{35}S]methionine in a cell-free rat liver system, was incubated with freshly isolated mitochondria for 30 min at 30°C. Labeled mitochondria (2.0 mg protein) were incubated at 37°C (final volume 0.8 ml) with: (▲) 0.23 ml rabbit reticulocyte lysate (115 mg/ml of protein), or (■) 0.3 M buffered sucrose.

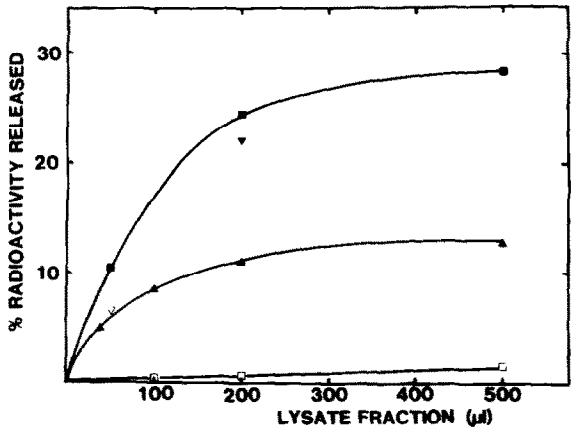

Fig.2. Release of mitochondrial proteins by the reticulocyte lysate fraction obtained by precipitation with ammonium sulphate. Labeled mitochondria (2.1 mg protein) were incubated at 37°C for 20 min (final volume 0.8 ml) with: (■) a fraction obtained by precipitation with ammonium sulphate at 55% saturation of reticulocyte lysate (FRL_{55}) (50 mg/ml protein); (▲) complete reticulocyte lysate (160 mg/ml protein); (□) supernatant of the precipitation with ammonium sulphate (100 mg/ml protein); (▽) FRL_{55} plus 0.15 ml supernatant fraction; (▼) FRL_{55} plus 1 mM salicylhydroxamic acid. In this figure as well as in figs 3 and 4, the value corresponding to the observed release of mitochondrial proteins in the isolation medium under the same incubation conditions has been subtracted from the experimental value.

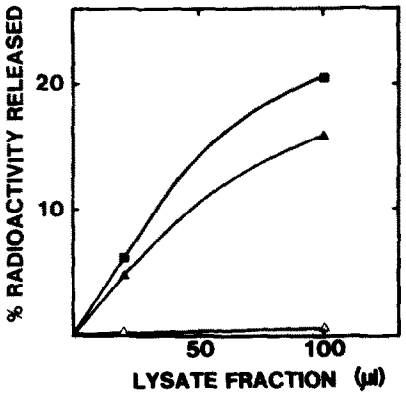

Fig.3. Effect of heat treatment of the lysate fraction obtained by precipitation with ammonium sulphate on the release of mitochondrial protein. Labeled mitochondria (2.0 mg protein) were incubated at 37°C for 20 min (final volume, 0.3 ml) with: (■) FRL_{55} (50 mg/ml protein); (▲) supernatant of FRL_{55} after 60°C, 5 min; (△) supernatant of FRL_{55} after 80°C, 5 min.

reticulocyte lipoxygenase on mitochondrial membranes by more than 90%. Moreover, neither succinate plus ADP nor KCN, which are also reported to inhibit lipoxygenase [10], inhibited the release (not shown). Thus, the lipoxygenase present in reticulocyte which can damage mitochondrial membranes [9–12], does not seem to be responsible for the observed release of proteins.

The activity of the ammonium sulphate fraction decreased after heating the fraction at 60°C for 5 min, and at 80°C was completely lost (fig.3). Moreover, since it is retained by a 10 kDa cutoff membrane (fig.4) it is probably protein(s).

The study of the transport of mitochondrial protein precursors has been carried out by in vitro incubation of mitochondria with the products synthesized in a cell-free system, mostly using the rabbit reticulocyte lysate [13]. Recently it was reported that the import of ornithine transcarbamoylase [14,15] and the β-subunit of mitochondrial ATPase [16] into mitochondria requires a reticulocyte protein(s) in addition to the precursor protein. In all cases the post-translational import has been studied but the exit of proteins from mitochondria under the experimental conditions has not been reported except in a brief communication [2]. Here, we show that the reticulocyte lysate, or a

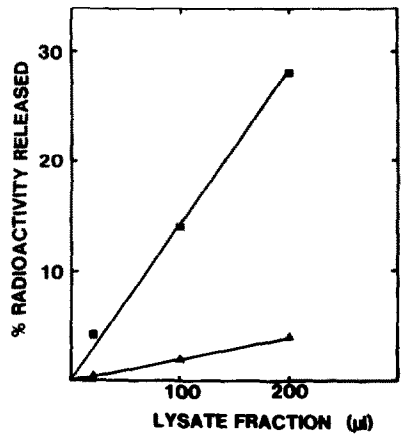

Fig.4. Effect of low and high molecular mass components from FRL_{55} on the release of mitochondrial protein. Labeled mitochondria (2.0 mg protein) were incubated at 37°C for 20 min (final volume, 0.55 ml) with (■) FRL_{55} retained by a 10 kDa cutoff membrane; (▲) FRL_{55} not retained by a 10 kDa cutoff membrane.

fraction thereof stimulates the exit of ^{35}S-labeled proteins previously imported into mitochondria.

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