Precursors of mitochondrial proteins are degraded in the cytosol at different rates

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The stability of rat liver mitochondrial protein precursors in the cytosol was investigated. The precursors were synthesized in a post-mitochondrial supernatant from rat liver, and mitochondria were then added at different times. The amount and pattern of proteins incorporated were determined. The precursors have different rates of transport into mitochondria. Some precursors are stable in the cytosol while most are degraded rapidly, with a half-life of about 30 min at 30°C.

Protein precursor *Mitochondria* *Protein degradation*

1. INTRODUCTION

Most mitochondrial proteins are synthesized in the cytosol as larger precursors which are then imported into mitochondria [1,2]. Since mitochondrial proteins are continuously being synthesized and degraded, there must be mechanisms to regulate the level of mitochondrial proteins. Such mechanisms could operate at the level of synthesis, degradation or transport from cytosol to mitochondria. It has been proposed that the entry of precursors into mitochondria can regulate the release of mitochondrial proteins by a 'push-pull' mechanism [3]. Also, ATP can play some role in the liberation of mitochondrial protein [4] and hemin specifically inhibits transfer of the precursor of δ-aminolevulinate synthase into chick embryo liver mitochondria [5–7].

One factor that can be involved in mitochondrial protein turnover is the stability of mitochondrial protein precursors in the cytosol.

In this paper, we report the stability in the cytosol of rat liver mitochondrial protein precursors synthesized in a homologous cell-free system in the presence or absence of mitochondria. Addition of mitochondria at different times after synthesis revealed that stability in the cytosol is different for many precursors and that most precursors are not stable.

2. MATERIALS AND METHODS

The preparation of the cell-free protein synthesizing system, and optimum conditions for protein synthesis and transport into mitochondria have been described [8]. Incubations were at 30°C and for 30 min unless otherwise indicated. 100 μl portions of cold protein synthesizing mixture were added to Eppendorf tubes in an ice bath and completed with 6 μl of 100 mM succinate. 100 μl of a mitochondrial suspension, containing 4 mg of protein 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) were added to a set of tubes. To another set, 100 μl of medium A1 were added. All tubes were incubated with shaking for the time indicated in each experiment. Then the tubes were centrifuged for 2.5 min at 10000 × *g*. The supernatants were taken with an Eppendorf micropipette and mixed with 100 μl of the mitochondrial suspension which had been kept on ice. All tubes were then incubated for 30 min and centrifuged for 2.5 min at 10000 × *g*. All mitochondrial pellets were washed 5 times with

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medium A1 and finally were suspended in 100 μl of 10% SDS. The trichloroacetic acid precipitable radioactivity was counted. In some cases, a portion was reserved for SDS-polyacrylamide gel electrophoresis.

3. RESULTS AND DISCUSSION

Rat liver mitochondrial protein precursors were synthesized in a homologous rat liver cell-free system [8] which does not contain mitochondria. Mitochondria were added after different periods of synthesis, and incorporation of protein into mitochondria was quantified. As shown in fig.1, there was maximum entry at 15 min followed by a decrease, indicating that some precursors had been degraded in the cytosol before the addition of mitochondria.

To confirm this finding, mitochondria were added at the beginning and after 15 and 45 min of synthesis. As shown in fig.2, when mitochondria were added 15 min after synthesis there was a decrease in the entry of proteins into mitochondria. Moreover, the entry at 45 min was less than at

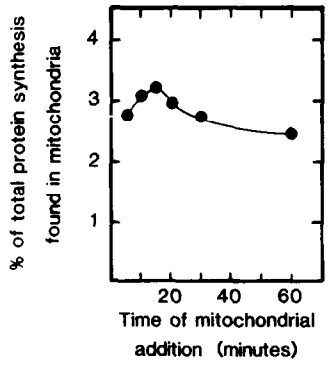

Fig.1. Effect of time of incubation on transport of mitochondrial protein precursors. Protein synthesis was carried out as described in [8]. After different times, 55 μl of the synthesis mixture were mixed in ice-cold tubes with 300 μl of 10 mM unlabeled methionine in medium A1. To each tube 20 μl of 100 mM succinate and 180 μl of a mitochondrial suspension containing 5 mg of protein were added. Tubes were incubated at 30°C for 30 min. Mitochondria were collected by centrifugation (2.5 min, 10000 × g) and washed 5 times with medium A1. Trichloroacetic acid precipitable radioactivity was measured in both mitochondria and supernatants.

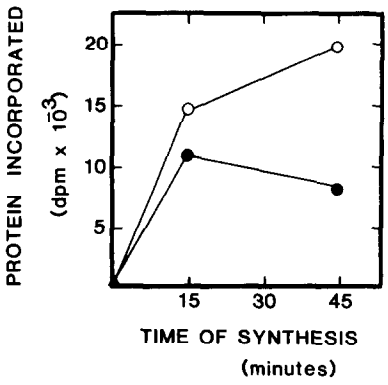

Fig.2. Effect of adding mitochondria at the beginning or after 15 and 45 min of protein synthesis, on import of mitochondrial protein precursors. Experiments were carried out as indicated in section 2. Mitochondria were added before (○) or after (●) protein synthesis which was allowed to proceed during the indicated times.

15 min, in contrast to the results when mitochondria were added at the beginning of synthesis.

The patterns of proteins incorporated into mitochondria are shown in fig.3. When mitochondria were added at the beginning of synthesis, at 15 min (fig.3A) two main protein peaks (1 and 2) appeared with M_r values of about 33000 and 38500 representing ~61% of the total protein incorporated. There is a low amount of proteins with an M_r higher than 38500. At 45 min (fig.3B), peaks 1 and 2 represented only 41% of the protein incorporated, and there was a considerable increase in the amount of proteins incorporated with a high M_r. Moreover, at 45 min the amounts of protein in peaks 1 and 2 were nearly the same, while at 15 min the amount of peak 2 was about twice that of 1.

The results obtained when mitochondria were added after 15 and 45 min of protein synthesis are shown in fig.3C and D. The import of proteins into mitochondria was clearly decreased, indicating that some precursors had been degraded in the cytosol before the addition of mitochondria. The patterns obtained indicate that some precursors are degraded faster than others. For example, import of peak 1, while still considerable at 15 min without mitochondria (fig.3C), is nearly abolished after 45 min. However, peak 2 seems to be more stable and some of it was still incorporated when mitochondria were added at 45 min (fig.3D).

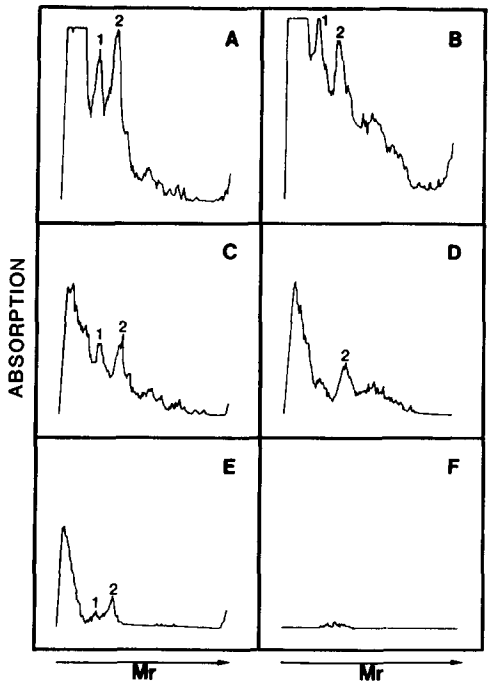

Fig.3. Stability of newly synthesized precursors in the cytosol. The experiment was carried out as described in section 2. Mitochondria were added before synthesis (A,B) or after synthesis (C,D,E and F). Protein synthesis was for 15 min (A,C and E) or for 45 min (B,D and F). For experiments shown in E and F the mitochondria were added to the post-mitochondrial supernatant obtained from the experiments shown in A and B. The mitochondrial pellets obtained were washed and subjected to SDS-polyacrylamide gel electrophoresis using 8% gels. Gels were treated for fluorography [9], Kodak X-Omat XS5 films were exposed, developed and subjected to densitometric scanning using an LKB 2202 ultroscan laser densitometer. Peaks 1 and 2 correspond to proteins with M_r values of about 33000 and 38500.

The presence of precursors in the post-mitochondrial supernatant after synthesis for 15 or 45 min in the presence of mitochondria (fig.3E and F) was also tested. It is shown that at 15 min there was a very low amount of precursors left, and most of the import corresponds to peak 2. At 45 min, obviously there were no precursors left since no import was detected. This confirms [10] that for most precursors the transport occurs immediately after synthesis. Possibly for some precursors (e.g. peak 2) the transport is slightly slower than the synthesis allowing accumulation of a small pool of it in the cytosol.

The above findings indicate that some precursors are more stable in the cytosol than others. It would then be expected that rat liver post-mitochondrial supernatants, when isolated, contain some precursors which could compete with those newly synthesized for entry into mitochondria.

As shown in fig.4, the post-mitochondrial supernatant competes with the transport of newly synthesized precursors. However, since 400 μl of post-mitochondrial supernatant produce a 50% inhibition of entry of labeled precursors formed by 66 μl of post-mitochondrial supernatant, the extract would contain roughly 16% of the precursors which it can synthesize under our standard conditions.

In summary, the results presented here show that many protein precursors have different rates of transport into mitochondria and that some of them are somewhat stable in the cytosol while most are degraded fairly fast, with an average half-life of about 30 min at 30°C.

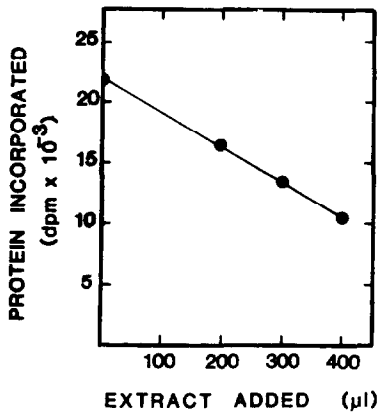

Fig.4. Effect of addition of post-mitochondrial supernatant on the import of newly synthesized precursors into mitochondria. Protein synthesis was carried out as described in [8]. 100 μl portions of the incubation were transferred to tubes containing the indicated amounts of unlabeled, untranslated post-mitochondrial supernatant containing 0.2 mg/ml cycloheximide. 20 μl of 100 mM succinate were added, and the volume of all samples was adjusted to 520 μl with medium A1. Then 100 μl of a mitochondrial suspension (2 mg of protein in medium A1) were added and the import assayed as in fig.1.

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