Adenosine 5′-triphosphate stimulates the release of polypeptides from mitochondria

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There was release of polypeptides to the medium when mitochondria containing labeled proteins were incubated with a rat liver post-mitochondrial supernatant. The release of polypeptides increased with the amount of rat liver extract added. Addition of cycloheximide did not inhibit the effect. Heating the post-mitochondrial supernatant did not inhibit the release of mitochondrial proteins, indicating that it was due to a heat-stable factor. The factor responsible has been isolated and identified as ATP. The presence of EDTA inhibits the release of polypeptides caused by ATP and Mg^{2+} stimulates it. The possible role of ATP in the turnover of mitochondrial proteins is briefly discussed.

Adenosine 5′-triphosphate *Mitochondria* *Protein turnover*

1. INTRODUCTION

Most mitochondrial proteins are synthesized in the cytosol as larger precursors [1–4] which are then imported into mitochondria by processes which are beginning to be clarified (review [5]) but little is known about degradation of mitochondrial proteins or coordination between their synthesis and degradation [6]. Recently, we showed that incubation of mitochondria containing labeled proteins with a reticulocyte lysate previously incubated under conditions of protein synthesis, accelerated the release of mitochondrial polypeptides to the medium [7]. Using similar conditions we found that a rat liver post-mitochondrial supernatant also induces release of mitochondrial polypeptides to the medium. Release of polypeptides was not inhibited by cycloheximide indicating that the effect was not due to the entry of 'in vitro' synthesized proteins but to factor(s) in the extract including, perhaps, precursors of mature proteins already present in the extract.

Here we describe the isolation and identification of ATP as the factor which appears to be responsible for the bulk of release of mitochondrial proteins under our experimental conditions.

2. MATERIALS AND METHODS

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

Rat liver extracts were prepared 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) according to Ogata et al. [8]; 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 (1200 Ci/mmol, 11 mCi/ml) and a mixture of the remaining 19 amino acids (50 μM each). Incubations were for 60 min at 30°C.

2.2. *Radioactive labeling of mitochondria*

Mitochondria were labeled either by incubation of hepatocytes with [^{35}S]methionine [7] or in vitro as follows: rat liver extracts (100 μl) that had synthesized proteins labeled with [^{35}S]methionine were

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incubated at 30°C for 30 min with mitochondria in 300 μl medium A1 containing 2 mM succinate. Samples were centrifuged at 10000 × *g* for 2 min and mitochondria were washed 6 times with 1 ml medium A1.

2.3. *Purification of the factor inducing the release of polypeptides from mitochondria. Gel filtration on Sephadex G-50 fine*

The rat liver extract (16 ml) was heated for 5 min at 100°C, cooled for 10 min in ice and centrifuged at 10000 × *g* for 8 min. The supernatant was lyophilized and brought to 2 ml with water. 1.8 ml were applied to a column (1.5 × 48 cm) of Sephadex G-50 fine equilibrated with medium B (medium A1 diluted 10 times with water). The column was eluted with the same buffer at a flow rate of 25 ml/h. Fractions of 6 ml were collected, lyophilized and brought to 0.6 ml with water and the release of polypeptides from mitochondria produced by each fraction was assayed.

2.4. *Determination of ATP*

ATP was determined enzymatically [9].

3. RESULTS AND DISCUSSION

3.1. *Incubation of mitochondria with the rat liver extract induces a release of polypeptides to the medium*

As already shown incubation of mitochondria with a reticulocyte lysate containing newly synthesized proteins accelerates the release of mitochondrial polypeptides to the medium. Although this release was not the result of gross physical disruption of mitochondria [7], to minimize possible artefacts due to the mitochondria degrading system present in reticulocytes we tested a rat liver post-mitochondrial supernatant. As shown in fig.1 the rat liver extract also produces a release of polypeptides. Fig.2 shows that the release (between 4 and 20% of mitochondrial labeled polypeptides) increases with the amount of rat liver extract added.

3.2. *The effect is not dependent on in vitro synthesized proteins*

To assess whether the effect is due to the entry into mitochondria of in vitro synthesized protein precursors, controls were carried out in which

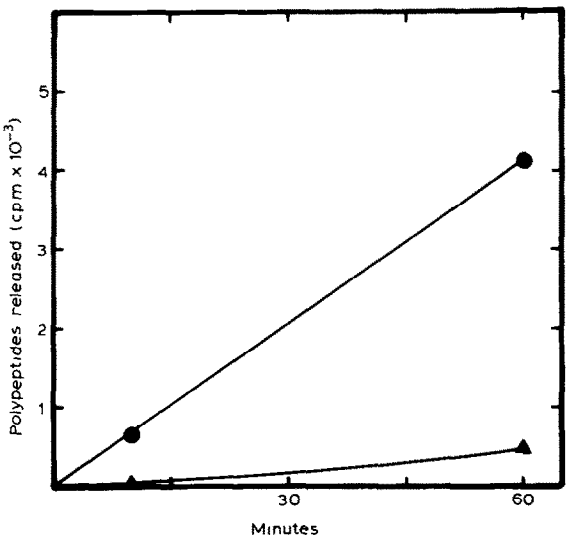

Fig.1. Effect of addition of rat liver post-mitochondrial supernatant on the release of trichloroacetic acid-precipitable material from mitochondria. Rat liver mitochondria (4 mg protein) labeled with [^{35}S]methionine were divided into two aliquots; each was resuspended in 300 μl medium A1, to one was added 100 μl medium A1 (▲) and to the other 100 μl unlabeled rat liver extract (●). 200-μl portions were taken at 10 and 60 min, mitochondria were pelleted and trichloroacetic acid-precipitable radioactivity in the medium was counted.

cycloheximide was added to inhibit protein synthesis. As shown in fig.3A the effect was the same with or without cycloheximide, indicating that it is produced by factor(s) preexisting in the extract. Fig.3 also shows that the factor(s) is depleted by preincubation of the extract with mitochondria. The release of polypeptides from mitochondria is reduced with one and nearly abolished by a second preincubation with fresh mitochondria. Fig.3 illustrates these experiments with mitochondria labeled in vitro (A) or by incubation of hepatocytes with [^{35}S]methionine (B).

3.3. *The effect is produced by ATP*

The stability of the factor to heat was studied. The factor was stable at 100°C suggesting a low M_r. The extract was heated for 5 min at 100°C, cooled in ice and centrifuged for 5 min at 10000 × *g*. The supernatant was subjected to gel filtration on Sephadex G-50 fine. The elution volume was equal to the volume of the gel bed, indicating that

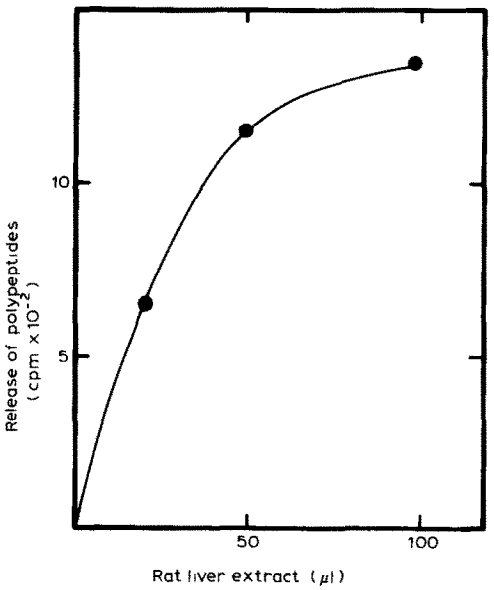

Fig.2. Effect of the amount of rat liver extract on the release of trichloroacetic acid-precipitable material from mitochondria. Labeled mitochondria (1 mg protein) were incubated for 30 min at 30°C with medium A1 or with different amounts of rat liver extract. Final volume of all samples was brought to 500 μl with medium A1. Mitochondria were pelleted and trichloroacetic acid-precipitable radioactivity in both mitochondria and medium was determined. The values had been corrected for experiments without extract (see fig.1).

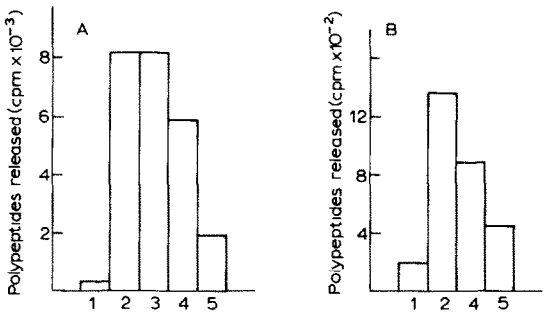

Fig.3. Effect of cycloheximide and of preincubation with mitochondria on the release of polypeptides from mitochondria. Mitochondrial proteins were labeled in vitro (A) or in cultured hepatocytes (B) as described in section 2. Well washed portions of labeled mitochondria were incubated at 30°C as in fig.2 with: (1) medium A1; (2) translated rat liver extract; (3) rat liver extract translated in the presence of 200 μg/ml cycloheximide (96% of protein synthesis inhibition); (4) rat liver extract preincubated with mitochondria once; (5) rat liver extract preincubated with mitochondria twice. After 30 min incubation, mitochondria were pelleted and trichloroacetic acid-precipitable radioactivity in the supernatant was counted. For preincubation with mitochondria rat liver extract was translated in the presence of cycloheximide (200 μg/ml) and preincubated for 30 min at 30°C with unlabeled mitochondria (1.3 mg protein). Mitochondria were pelleted by centrifugation and the supernatant was incubated with labeled mitochondria as described above. For sample 5 the supernatant of the first preincubation was subjected to a second preincubation before incubation with labeled mitochondria. During preincubation all 5 samples were incubated at 30°C.

their M_r is lower than 1500. Therefore, the fraction containing the factor was fractionated by gel filtration on Sephadex G-10. The elution profile is shown in fig.4.

The absorption spectra of the fraction containing the maximum of activity was very similar to that of ATP in the same medium (fig.4); the absorption peak was at 261 nm in both cases and shifted like ATP when the sample was made 0.1 N in HCl. These results suggested that the active fraction contains mainly ATP.

Fig.5 shows that addition of pure ATP indeed induces a release of polypeptides from mitochondria to the medium. The effect produced increases up to about 5 mM ATP.

We checked if the ATP present in the extract can account for all the effect. The ATP determined enzymatically was 0.67 mM. Since rat liver cytosol contains 3 mM ATP [10], as the liver was homogenized in 2.5 vols of medium, the ATP measured agrees with the expected value. The effect produced by the supernatant was compared with that produced by ATP. The results shown in fig.6 indicate that ATP in the sample can account for all the effect produced.

Table 1 shows that GTP and UTP are active. However, the concentrations of these nucleotides in rat liver cytosol are much lower than ATP [10,11].

Most reactions involving ATP require Mg^{2+}. Thus we investigated if Mg^{2+} is required for the release of polypeptides. Addition of 2 mM EDTA reduces the effect of 100 μM ATP by 62%. Without addition of EDTA and Mg^{2+} the effect is 80% of the maximum which is reached with 50 μM Mg^{2+}. Higher concentrations of Mg^{2+} slightly in-

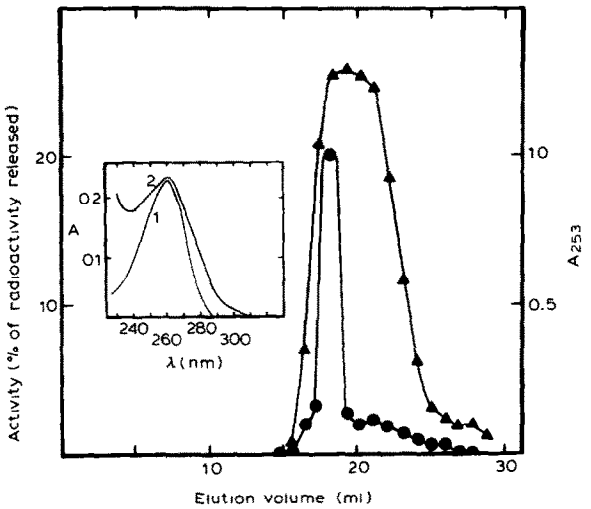

Fig.4. Purification of the factor inducing the release of polypeptides from mitochondria; gel filtration on Sephadex G-10. The fraction eluted from the Sephadex G-50 fine column showing higher activity was applied to a column (1.5 × 29 cm) of Sephadex G-10 and eluted with medium B (medium A1 diluted 10 times with water) at a flow rate of 14 ml/h. Fractions of 1 ml were collected, lyophilized and brought to 0.1 ml with water to reconstitute the medium A1. The release of polypeptides from mitochondria produced by each fraction was assayed as in fig.2. As a reference of the elution, the absorption of each fraction at 253 nm (▲—▲) was measured. The activity (●—●) is expressed as percent of radioactivity of mitochondrial polypeptides released into the medium. (Inset) Absorption spectra of the active fraction and of ATP. To obtain spectrum 1, 100 μl of 0.1 mM ATP in medium A1 were diluted to 1 ml with water. To obtain spectrum 2, the fraction from the Sephadex G-10 column showing highest activity was diluted 10 times with bidistilled water. Spectra were measured at 22°C in a Bausch and Lomb Spectronic 2000 spectrophotometer.

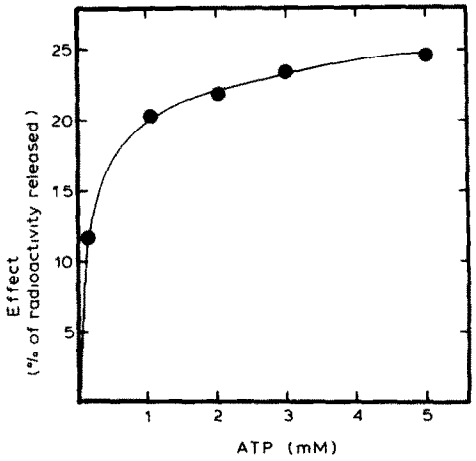

Fig.5. Release of polypeptides from mitochondria produced by ATP. Labeled mitochondria (1 mg protein) were incubated for 30 min at 30°C with different concentrations of ATP in medium A1 in a final volume of 500 μl. Mitochondria were pelleted by centrifugation (10000 × g, 5 min) and trichloroacetic acid-precipitable radioactivity in both mitochondria and supernatant was counted. The activity is expressed as percent of radioactivity of mitochondrial polypeptides released into the medium.

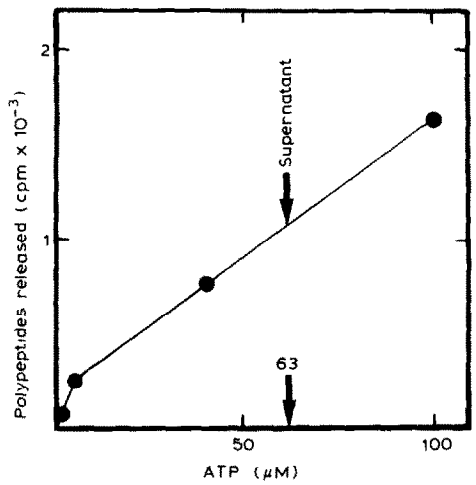

Fig.6. The amount of ATP present in the supernatant of heating the extract at 100°C can account for all the effect produced. The rat liver extract was heated for 5 min at 100°C, cooled for 10 min in ice and centrifuged at 10000 × g for 8 min. The ATP in the supernatant was determined enzymatically to be 0.67 mM. The release of polypeptides from mitochondria produced by 50 μl of the supernatant in a final volume of 500 μl was assayed as in fig.2 and is indicated by arrows. The effect produced by 2,5,40 and 100 μM ATP was also assayed. The supernatant (containing 67 μM final ATP) had an effect equivalent to that of 63 μM ATP.

hibited the effect which is 77.2% of maximum with 5 mM Mg^{2+}. The activity without addition of Mg^{2+} possibly reflects the Mg^{2+} present in mitochondria.

The mechanism by which ATP induces the release of polypeptides from mitochondria is not known; however, several hypotheses can be advanced as follows: (i) phosphorylation of membrane proteins; (ii) phosphorylation within mitochondria of polypeptides to be released; (iii) activation of ATP-dependent mitochondrial pro-

Table 1

Release of polypeptides from mitochondria produced by different substances

Substance	Polypeptides released (cpm)
ATP	1624
GTP	1138
UTP	858
$NADP^+$	0
Amino acids[a]	14
ATP + 2 mM EDTA	662

[a] A mixture of 20 amino acids (50 μM each)

Labeled mitochondria (1 mg protein) were incubated for 30 min at 30°C with 100 μM final concentration of each substance in medium A1 in a final volume of 500 μl. Mitochondria were pelleted by centrifugation (10000 × *g*, 5 min) and trichloroacetic acid-precipitable radioactivity in the supernatant was counted

tease; (iv) to maintain the membrane potential in mitochondria [12,13] and this potential regulates the release of polypeptides.

In summary, we have shown that a rat liver post-mitochondrial supernatant produces a release of polypeptides from mitochondria, that this effect is produced by a factor preexisting in the rat liver extract, and that this factor is ATP. We have suggested, on the basis of experimental evidence, that a push-pull mechanism may play an important role in mitochondrial protein turnover [7]. While the present experiments clearly demonstrate liberation of mitochondrial polypeptides by ATP they should not be construed as negating the push-pull hypothesis but rather as identifying a possibly crucial factor in an obviously complex process. Experiments to determine the mechanism of action of ATP and its possible role in the regulation of mitochondrial protein turnover are now needed.

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