# EVIDENCE FOR A TRANSLATIONAL INHIBITOR ISOLATED FROM RAT LIVER MITOCHONDRIA

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## 1. Introduction

Previous studies have shown that the organelle mitochondria contain an active intrinsic protein synthesizing system which may be functionally and physically distinguished from the cytoplasmic translational components. Evidence has accumulated that biogenesis of the functional inner mitochondrial membrane requires the coordination of mitochondrial and nuclear genetic apparatus [1,2]. For example, the biosynthesis of different structural polypeptides of the respiratory electron transport by the mitochondrial and cytoplasmic translation systems has been suggested to be regulated through cellular control mechanisms, the nature of which remain to be determined [3,4]. The role of nuclear gene products as effectors of mitochondrial translation and/or transcription is further supported by genetic studies [1–4]. Mitochondrial translational products are typically represented by 8–10 hydrophobic proteins which are associated with enzymes of the respiratory chain, such as oligomycin-sensitive ATPase [5,6], cytochromes $a$–$a_3$ [7,8] and cytochrome $b$ [9,10]. A nuclear gene product appears to exert an effect on polypeptides synthesized on mitochondrial ribosomes [11]. At least one cytoplasmic protein may specifically stimulate the synthesis of subunits I–III of cytochrome $c$ oxidase [12]. They suggested further that the cessation of mitochondrial protein synthesis in vitro after short periods of incubation may be attributed to the depletion of essential cytoplasmic components [12]. Here, we describe the isolation of an inhibitor of protein synthesis from rat liver mitochondria. We also report on the conditions which allow for the activation of this inhibitor in vitro.

## 2. Materials and methods

Chemicals and biological compounds of the highest purity were purchased from Sigma. PL Biochemicals, and Boehringer-Mannheim. L-[U-$^{14}$C]-Leucine (356 Ci/mol) was from New England Nuclear Corp. and DEAE-cellulose (DE-22) was from Whatman. Rabbit reticulocyte lysates were obtained from Clinical Convenience Products (Madison, WI) and were gifts from Dr Robert J. Suhadolnik (Department of Biochemistry, Temple University), and Drs Rosemary Jagus and Brian Safer (National Institutes of Health, Bethesda, MD). Cell-free protein synthesis assays were done with the addition of creatine phosphate (915 mM) and creatine phosphokinase (45 units/ml) as in [13–15]. The hemin was 12.5 μM and leucine was added to 100 μM final con. (spec. act. 30 cpm/pmol). Aliquots (7.5 μl) were removed at indicated times and processed for incorporation leucine into polypeptides [13,14]. Rat liver mitochondria were prepared from Sprague-Dawley rats by the methods in [16] and conditions for protein synthesis in isolated mitochondria were as in [17].

### 2.1. *Preparation of rat liver mitochondrial extract*

Each mitochondrial preparation (from rats fasted for 48 h) was suspended in 3 ml 10 mM Hepes/KOH, 10 mM KCl, 1.5 mM MgOAc, 0.5 mM dithiothreitol, 0.5% NP 40 (Calbiochem), pH 7.5 and left on ice for 10 min. The mitochondrial suspension was homogenized with 10–15 strokes in a Dounce homogenizer. The homogenate was centrifuged at 12 000 × $g$ for 20 min. The clear supernatant was removed (care was exercised not to disturb the cloudy top layer), divided into 0.7 ml aliquots and kept at −40°C. The

mitochondrial extract has an $A_{280}$ of 30.3/ml (av. 3 prep.) corresponding to 18.5 mg protein/ml (determined using the Bio-Rad protein assay kit with bovine globulin as the protein standard).

2.2. *Treatment of rat liver mitochondrial extract with N-ethyl maleimide (NEM)*

Crude mitochondrial extract (20 μl) was incubated with 100 mM NEM (2 μl) for 0 or 10 min, 37°C followed by the addition of 100 mM dithiothreitol (3 μl). The total reaction mixture was further incubated on ice for 10 min. Buffer (50 μl) (20 mM Hepes/KOH, 1 mM dithiothreitol (pH 7.5)) was added to the NEM-treated mitochondrial extract, followed by the addition of an equal volume of ice-cold glass distilled water (75 μl). Aliquots of the diluted, NEM-treated mitochondrial extract were then tested for protein synthesis inhibitory activity using rabbit reticulocyte lysates.

2.3. *DEAE-cellulose column chromatography*

Crude mitochondrial extract (0.7 ml) was applied to a column of DEAE-cellulose (1 × 5 cm) equilibrated with buffer A (20 mM Tris–HCl, 1 mM dithiothreitol, 1 mM $MgCl_2$ (pH 7.6)) containing 20 mM KCl. Five 2 ml fractions were collected. The column was further eluted with buffer A containing 250 mM KCl and five 2 ml fractions were collected. The column was then eluted with buffer A containing 750 mM KCl and another five 2 ml fractions were collected. Aliquots of each fraction (20 μl) were activated with NEM for 0 or 10 min as in section 2.2. Following neutralization of NEM with dithiothreitol, aliquots (3 μl) of the activated fractions were tested for their ability to inhibit polypeptide synthesis using rabbit reticulocyte lysates. The first 3 fractions of the 250 nM eluate (see fig.1) were pooled and concentrated with solid ammonium sulfate (0–70% saturation). The precipitate was dissolved in buffer B (10 mM Hepes, 50 mM KCl, 1 mM dithiothreitol, 0.05 mM EDTA, 10% glycerol (pH 7.5) dialyzed overnight against the same buffer and stored in small aliquots, either at −40°C or in liquid nitrogen.

## 3. Results

The specific inhibitor of mitochondrial protein synthesis, chloromphenicol, resulted in a 94% inhibition of [U-$^{14}$C]leucine incorporation into isolated rat liver mitochondria protein. In contrast, cycloheximide, a specific inhibitor of protein synthesis on cytoplasmic ribosomes had no effect (table 1). These results clearly demonstrated that our mitochondrial preparations were minimally contaminated by cytoplasmic ribosomes (2–5%). The addition of an S-100 fraction from rat liver stimulated mitochondrial protein synthesis by 20% (table 1).

Table 1
Effect of inhibitors of protein synthesis on [U-$^{14}$C]leucine incorporation by isolated rat liver mitochondria

| Conditions | [Inhibitor] (μM) | cpm/mg incorp. | Inhibn. (%) |
|---|---|---|---|
| Control | 0 | 13 476 | 0 |
| Chloramphenicol | 0.31 | 915 | 94 |
| Cycloheximide | 3.5 | 13 535 | 0 |
| S-100 Fraction (0 5 mg) | | 16 299 | −21 |

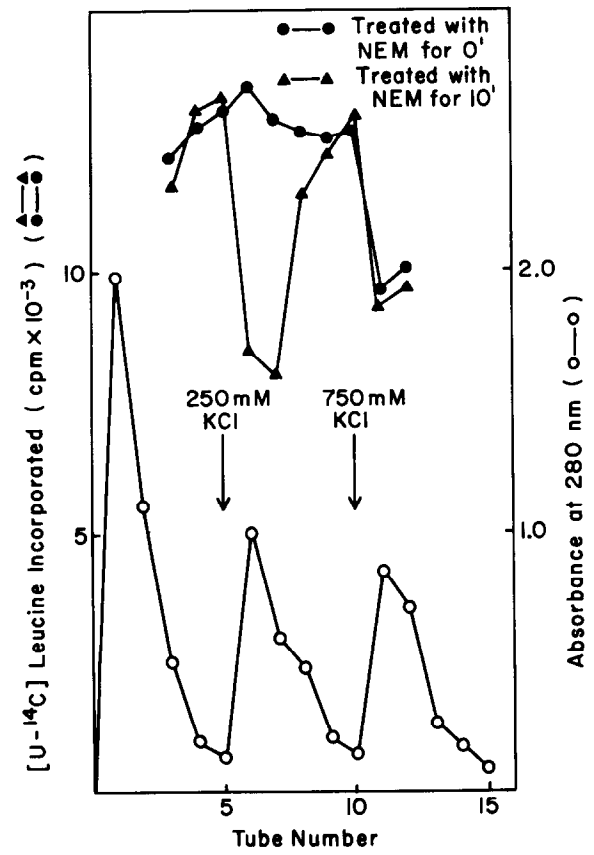
Incubation in duplicate was for 30 min at 37°C in the media of section 2. Duplicates were within 10% of each other. Each value was corrected by subtracting a zero time value

Because protein synthesis in isolated rat liver mitochondria ceased after 20 min incubation in vitro (not shown), we investigated the possibility that an inhibitor of polypeptide synthesis might be associated with the isolated rat liver mitochondria and which might become activated in vitro. Pilot studies showed that the rabbit reticulocyte lysate translating system could be used as a reliable and sensitive assay for the activity of a translational inhibitor isolated from rat liver mitochondria. When non-treated mitochondrial extract was added to a rabbit reticulocyte cell-free protein synthesizing system, little or no inhibition of polypeptide synthesis was observed (table 2). However, when the mitochondrial extract was incubated with NEM for 10 min, and the NEM subsequently neutralized with excess dithiothreitol, the treated mitochondrial extract resulted in a 40–60% inhibition of polypeptide synthesis in reticulocyte lysate (table 2). When a sample of non-treated mitochondrial extract was passed through a DEAE-cellulose column and the column sequentially eluted with buffer containing increasing KCl concentrations (20 mM KCl, followed by 250 mM and 750 mM KCl), 3 protein peaks were resolved (fig.1). Fractions of the 250 mM KCl eluate were individually treated with NEM, then tested for inhibitory activity in the reticulocyte lysate translating system. Before NEM treat-

Table 2
Effect of *N*-ethyl maleimide (NEM) on inhibition of polypeptide synthesis in rabbit reticulocyte lysates by rat liver mitochondrial extracts

| Addition | [U-$^{14}$C]leucine incorporated into protein[a] | | | | |
|---|---|---|---|---|---|
| | NEM[b] (0 min) | | | NEM (10 min) | |
| | 0 μl[c] | 2 μl | 4 μl | 2 μl | 4 μl |
| Control | 11 200 | – | – | – | – |
| Plus mitochondrial extract | | | | | |
| Preparation 1 | – | 11 310 | 10 050 | 6870 | 4980 |
| Preparation 2 | – | 12 540 | 11 570 | 7050 | 4720 |
| Preparation 3 | – | 11 730 | 10 890 | 6940 | 5270 |

[a] Incubations were at 30°C, 60 min. The incorporation of [U-$^{14}$C]leucine was measured by removing 7.5 μl aliquots
[b] NEM treatment was performed as in section 2
[c] μl extract added/30 μl assay




ment, fractions eluted with 250 mM KCl showed little inhibitory activity. Following treatment with NEM, a distinct inhibitory peak, which coincided with the $A_{280}$ peak, became evident (fig.1). The peak inhibitory fractions were concentrated with ammonium sulfate (0–70%) and added to reticulocyte lysates at varying concentrations. The inhibitory activity was apparent only after 5 min incubation and reached a maximum after 30–45 min (fig.2).

## 4. Discussion

These experiments indicate that the isolated and purified rat liver mitochondria contain a latent inhibitor of polypeptide synthesis which can be activated by incubation with *N*-ethyl maleimide. This inhibitor may account for at least part of the cessation of mitochondrial protein synthesis observed in vitro. Whether mitochondrial translation is affected by this inhibitor remains to be determined.

Partially purified mitochondrial inhibitor has no effect on the initial rate of leucine incorporation into

Fig.1. Elution of rat liver mitochondrial inhibitor from DEAE-cellulose. Conditions for elution were as in section 2. Activation of inhibitor with *N*-ethyl maleimide and assay for inhibitory activity using rabbit reticulocyte lysates were as in table 2. Inhibitions in fractions eluted with 750 mM KCl are due to the high salt present in the buffer.

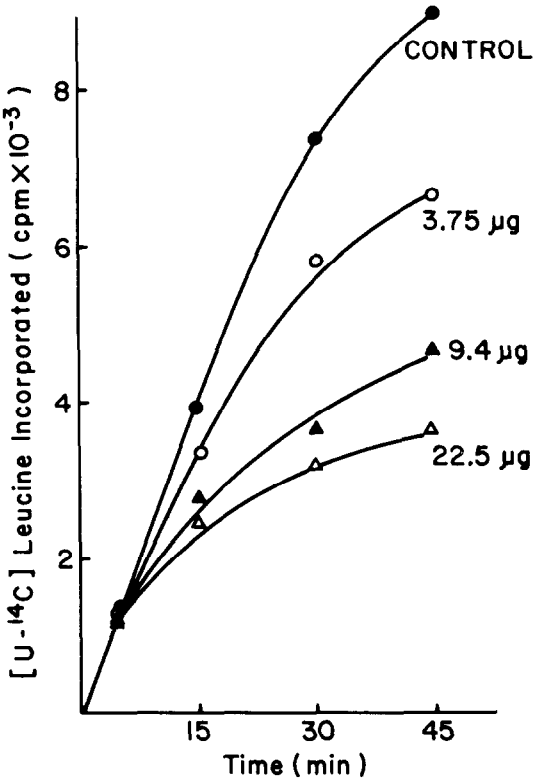



Fig.2. Effects of increasing concentrations of partially purified rat liver mitochondrial inhibitor on kinetics of protein synthesis in rabbit reticulocyte lysates. Conditions for polypeptide synthesis were as in section 2. (●) Control; (○) 3.75 µg inhibitor/30 µl assay; (▲) 9.4 µg inhibitor; (△) 22.5 µg inhibitor.

polypeptides, but results in a marked decrease in the rate of protein synthesis at later times of incubation (fig.2). This suggests that the inhibitor may act specifically at some as yet undetermined step in the initiation process. It is also interesting to note that the mitochondrial inhibitor described here appears to have activation and elution properties similar to an extensively studied hemin-regulated translational inhibitor isolated from the post-ribosomal supernatant of reticulocyte lysates [17–20]. A logical extension of these studies would be to determine the exact site of action of this inhibitor in lysed rabbit reticulocyte and to study its effect on mitochondrial translation. These studies are under investigation in this laboratory.

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## References

[1] Beattie, D. S. (1971) Subcell. Biochem. 1, 1–23.
[2] Buetow, D. E. and Wood, W. M. (1978) Subcell. Biochem. 5, 1–85.
[3] Barath, Z. and Kuntzel, H. (1972) Proc. Natl. Acad. Sci. USA 69, 1371–1374.
[4] Ibrahim, N. G. and Beattie, D. S. (1976) J. Biol. Chem. 251, 108–115.
[5] Tzagoloff, A., Akai, A., Needleman, R. B. and Zulch, G. (1975) J. Biol. Chem. 250, 8236–8242.
[6] Slonimski, P. P. and Tzagoloff, A. (1976) Eur. J. Biochem. 61, 27–41.
[7] Nargang, F. E., Bertrand, H. and Werner, S. (1979) Eur. J. Biochem. 102, 297–307.
[8] Chen, W. L. and Charalampous, F. C. (1969) J. Biol. Chem. 244, 2767–2776.
[9] Lin, L. F. H., Kim, I. C. and Beattie, D. S. (1974) Arch. Biochem. Biophys. 160, 458–464.
[10] Weiss, H. (1972) Eru. J. Biochem. 30, 469–478.
[11] Ono, B., Fink, G. and Schatz, G. (1975) J. Biol. Chem. 250, 775–782.
[12] Poyton, R. O. and Kavanagh, L. (1976) Proc. Natl. Acad. Sci. USA 73, 3947–3950.
[13] Wu, J., Cheung, C. and Suhadolnik, R. J. (1978) Biochem. Biophys. Res. Commun. 82, 921–928.
[14] Wu, J., Cheung, C. and Suhadolnik, R. J. Biol. Chem. 253, 7295–7300.
[15] Wu, J., Cheung, C. P., Bruzel, A. R. and Suhadolnik, R. J. (1979) Biochem. Biophys. Res. Commun. 86, 648–653.
[16] Beattie, D. S., Basford, R. E. and Koritz, S. B. (1967) J. Biol. Chem. 242, 3366–3368.
[17] Ibrahim, N. G., Burke, J. B. and Beattie, D. S. (1974) J. Biol. Chem. 249, 6806–6811.
[18] Ranu, R. S. and London, I. M. (1976) Proc. Natl. Acad. Sci. USA 73, 4349–4353.
[19] Safer, B. and Anderson, W. F. (1978) CRC Crit. Rev. Biochem. 5, 261–290.
[20] Gross, M. (1979) J. Biol. Chem. 254, 2370–2377.