

## CYCLOHEXIMIDE RESISTANT AMINO ACID INCORPORATION INTO MITOCHONDRIAL PROTEIN FROM *NEUROSPORA CRASSA* *IN VIVO*

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### 1. Introduction

In experiments with flight muscle of *Locusta migratoria*, a cycloheximide-resistant mitochondrial protein synthesis has been demonstrated *in vivo* [1]. This cycloheximide resistant amino acid incorporation into fractions of mitochondrial membrane proteins separated by electrophoresis has been compared with incorporation by isolated mitochondria *in vitro*. Similar labelling patterns have been obtained. In the present communication, these results are confirmed and extended in experiments with growing mycelia from *Neurospora crassa*.

### 2. Methods

*Neurospora crassa* wild type (strain 74 A) hyphae were grown for 18 hr under conditions described previously [2]. For each point in figs. 1 and 2, one 50 ml culture was taken. Mitochondria were isolated by grinding the washed hyphae mechanically between two corundum discs [8] in a sucrose medium (0.44 M sucrose, 1 mM EDTA) [3] containing the unlabelled amino acids (10 mM), which were used as labelled precursors in the experiment. The homogenate was centrifuged 10 min at 1,500 X g. Mitochondria were isolated from the supernatant by centrifuging 30 min at 8,000 X g, resuspended in sucrose medium and purified by repeating both centrifugations [4]. The postmitochondrial supernatant was centrifuged for 30 min at 20,000 X g yielding the 20,000 X g supernatant containing ribosomal and cell sap proteins. Methods for determining specific radioactivity, pre-

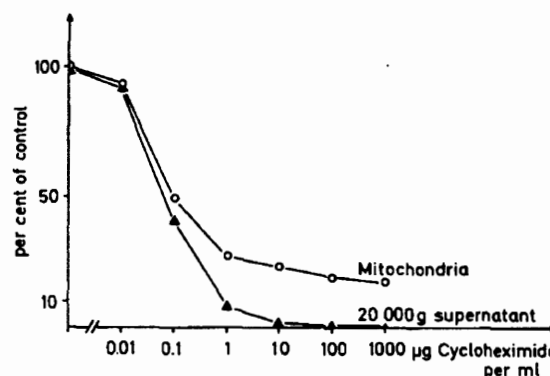


Fig. 1. *In vivo* incorporation of  $^{14}\text{C}$ -L-leucine (10 nCi/ml) into the protein of whole mitochondria (o—o) and of 20,000 X g supernatant (▲—▲) in the presence of different concentrations of cycloheximide. The values represent percentage of control without cycloheximide. Cycloheximide was added 5 min prior to a 10 min labelling period.

paration of insoluble mitochondrial protein, electrophoretic separation and quantitative evaluation of radioactivity in the pherograms have been described [1]. Mitochondria were labelled *in vitro* in a medium described recently [2]. Cycloheximide was purchased from Sigma Chemical Company, and U- $^{14}\text{C}$  L-amino acids from the Radiochemical Centre, Amersham (leucine 305 mCi/mole, isoleucine 311 mCi/mole and phenylalanine 459 mCi/mole).

### 3. Results

In the presence of 100 µg cycloheximide per ml, the radioactivity incorporated into proteins of the

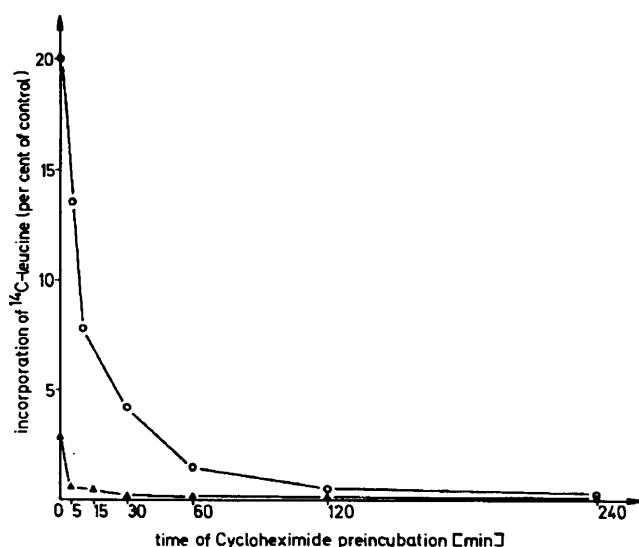


Fig. 2. *In vivo* incorporation of <sup>14</sup>C-L-leucine (10 nCi/ml) into the protein of whole mitochondria (○—○) and 20,000 X g supernatant (▲—▲) for different times of cycloheximide preincubation (100 μg/ml). The values represent percentage of control without cycloheximide. The labelling period was 10 min.

20,000 X g supernatant is less than 1% of control, but is 15–20% with whole mitochondrial protein (fig. 1). Thus, incorporation of amino acids into the proteins of the cytosol is inhibited to a higher extent than that into the mitochondrial proteins.

The radioactivity incorporated during a 10 min period decreases with increasing cycloheximide preincubation (fig. 2). However, incorporation is still observed following preincubation up to 2 hr, whereas after 4 hr an incorporation into proteins both from the cytosol and the mitochondria is undetectable.

Mitochondria isolated from hyphae preincubated *in vivo* for 4 hr with cycloheximide incorporate *in vitro* about 50% of the <sup>14</sup>C-amino acids as compared with mitochondria isolated from untreated hyphae (fig. 3). Thus, the mitochondrial protein-synthesizing system is not irreversibly damaged following even prolonged cycloheximide preincubation. In the experiments shown in fig. 3, 5 μg cycloheximide per ml were used. Similar results are obtained with higher concentrations.

Amino acid incorporation into extractable mitochondrial proteins is inhibited by cycloheximide to

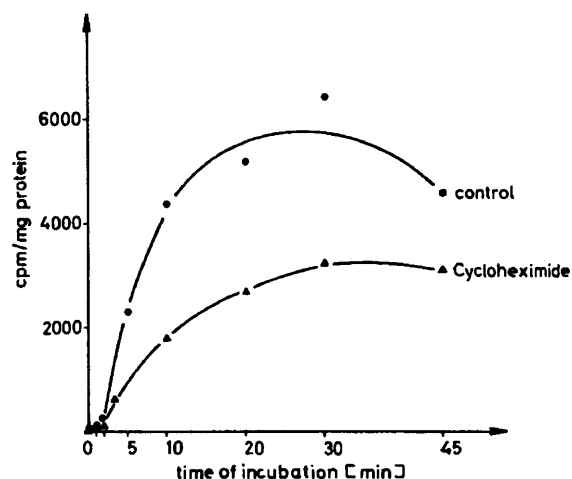


Fig. 3. Incorporation of <sup>14</sup>C-L-leucine into mitochondria *in vitro*. Control: mitochondria isolated from untreated hyphae. Cycloheximide: mitochondria from hyphae which were incubated for 4 hr with cycloheximide (5 μg/ml) prior to isolation.

the same extent (97%) as incorporation into proteins of the cytosol. Cycloheximide-resistant incorporation is found almost exclusively in the mitochondrial membrane proteins. This fraction still shows a specific radioactivity of 40% of the control (table 1).

The electrophoretic separation of mitochondrial membrane proteins (fig. 4) indicates that the radioactivity incorporated *in vivo* (control) is distributed almost proportionally to the amido black staining of the individual bands. The radioactivity incorporated *in vivo* under cycloheximide action is found to be highest in fractions 11 and 18, while minor radioactivity is concentrated in fractions 8, 10, 16 and 23. These fractions correspond to the protein bands 2, 3, 4, 6, 8 and 10 as defined previously [2].

#### 4. Discussion

A cycloheximide-resistant amino acid incorporation into mitochondrial protein from *Neurospora crassa* was recently reported also by Küntzel [5]. In addition, experiments presented in this paper show that only a few fractions of mitochondrial membrane proteins are synthesized by the intrinsic mitochondrial protein synthesis *in vivo*, confirming the results obtained by amino acid incorporation into isolated mito-

Table 1

Influence of cycloheximide on the *in vivo* incorporation of labelled amino acids into mitochondrial subfractions of *Neurospora crassa*. Hyphae in ten 50 ml cultures were labelled in parallel experiments for 30 min with  $^{14}\text{C}$ -L-leucine and  $^{14}\text{C}$ -L-leucine and  $^{14}\text{C}$ -L-phenylalanine, each 100 nCi/ml, followed by a 30 min chase with the corresponding unlabelled amino acids (2 mM). 5 cultures were preincubated with cycloheximide (100  $\mu\text{g}/\text{ml}$ ) for 5 min.

Cellular protein fraction	cpm/mg protein control	With cycloheximide
20,000 X g supernatant	130,000	3,000
Mitochondria	Whole	28,000
	Extractable	3,100
	Residual*	60,000

\* These fractions were used for gel electrophoresis (fig. 4).

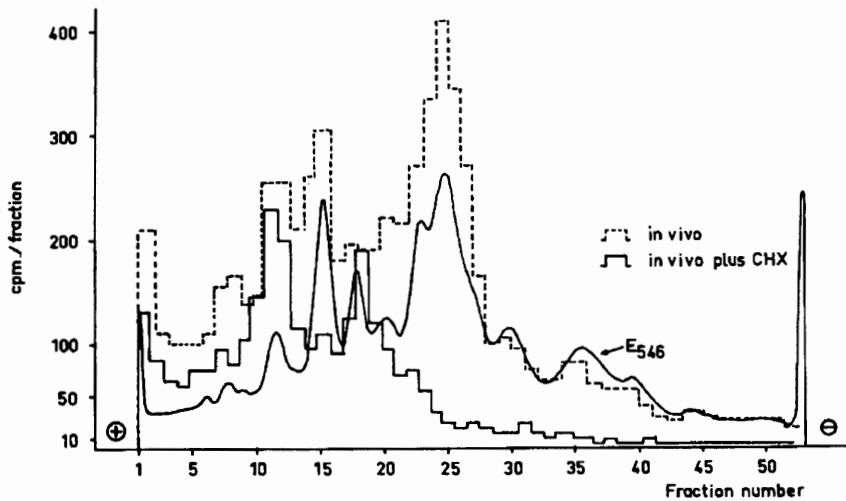


Fig. 4. Densitogram of amino black stained insoluble mitochondrial protein after gel electrophoresis (smooth line), and distribution of *in vivo* incorporated radioactivity (edged lines), in the presence (—) or absence (---) of cycloheximide (CHX) (100 mg/ml).

chondria [2]. In *in vivo* and *in vitro* experiments, after electrophoretic separation of membrane proteins, the highest specific radioactivity is found in a band of low mobility, previously called band 4 [2]. In mitochondria from flight muscle of *Locusta migratoria* and from *Neurospora*, the labelling pattern is similar, showing labelled bands nearly in the same positions after electrophoretic separation of the membrane proteins.

After the labelling of isolated mitochondria *in vitro*, incorporated amino acids are partially associated with mitochondrial ribosomes, as demonstrated recently by Neupert et al. [7]. This is not observed, however, when mitochondria are labelled *in vivo* under cyclo-

heximide action followed by a 30 min chase [6]. Hence, it may be assumed that the labelling pattern obtained *in vivo* in the presence of cycloheximide represents predominantly complete peptide chains.

It cannot be decided, however, if the peptide chains synthesized by the intrinsic mitochondrial system are integrated into functionally active proteins, when the extramitochondrial protein synthesis is eliminated simultaneously. This problem may be considered with experiments *in vitro* as well as *in vivo* under cycloheximide action. Nevertheless the mitochondrial protein synthesis is functioning for at least 1 hr after the elimination of the extramitochondrial system by cyclo-

heximide. This indicates that the two systems are not closely coupled or that the coupling is weakened by the action of the antibiotic.

The incorporation of  $^{14}\text{C}$ -leucine into mitochondrial proteins decreases with increasing cycloheximide preincubation (fig. 2). In preliminary experiments, however, an increased leucine pool was observed after inhibition of the main part of cellular protein synthesis by the antibiotic [6]. If the dilution of the added  $^{14}\text{C}$ -leucine by the pool is considered, it may be calculated that about 50% of the original mitochondrial protein synthesis persists after 45 min cycloheximide preincubation.

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#### References

- [1] W.Seald, Th.Hofstötter, D.Hacker and Th.Bücher, FEBS Letters 2 (1969) 177.
- [2] W.Seald, Th.Bücher, B.Olbrich and F.Kaudewitz, FEBS Letters 1 (1968) 235.
- [3] D.J.L.Luck, J. Cell Biol. 16 (1963) 483.
- [4] J.W.Greenawalt, D.O.Hall and O.C.Wallis, in: Methods of Enzymology, vol. 10, eds. R.W.Estabrook and M.E.Pullman (Academic Press, New York and London) pp. 142--147.
- [5] H.Küntzel, Nature 222 (1969) 142.
- [6] W.Seald, A. Schwab and Th.Bücher, 20th Mosbach Kolloquium, eds. Th.Bücher and H.Sies (Springer Verlag, Heidelberg) in press.
- [7] W.Neupert, W.Seald, A.J.Schwab, P.Massinger and Th. Bücher, European J. Biochem., submitted for publication.
- [8] H.Weiss, experiments to be published.