INCORPORATION OF AMINO ACIDS INTO MITOCHONDRIAL PROTEIN OF THE FLIGHT MUSCLE OF *LOCUSTA MIGRATORIA IN VITRO* AND *IN VIVO* IN THE PRESENCE OF CYCLOHEXIMIDE

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1. Introduction

The incorporation of amino acids into distinct protein fractions of mitochondria from various sources [1–4] including a cytoplasmic mutant of *Neurospora crassa* [5] has been shown to be an inherent capacity of isolated mitochondria *in vitro*. An independent method for studying this system is offered by the specific inhibition of extramitochondrial protein synthesis *in vivo*. Cycloheximide is known as a strong inhibitor of protein synthesis catalysed by microsomal ribosomes of higher organisms [6]. In contrast, protein synthesis in isolated mitochondria *in vitro* [3,5,7] similar to bacteria [8] is unaffected by the antibiotic. Some recent reports indicate furthermore that *in vivo* under the influence of cycloheximide, incorporation of amino acids into mitochondrial proteins is inhibited to a smaller extent than incorporation into microsomal proteins [9]. In this communication we report experiments *in vivo* with the flight muscle of *Locusta migratoria*. Under the influence of cycloheximide, amino acids are incorporated only into some fractions of electrophoretically separated mitochondrial proteins. The labelling pattern is very similar after incorporation *in vitro*, but clearly distinct in a control experiment *in vivo* without cycloheximide.

2. Methods

Throughout the experiments 12-hour starved locusts were used 2 days after the imaginal moulting [11]. Cycloheximide (Sigma, Chemical Company) and 14-C-1-amino acids (u) (Radiochemical Centre, Amersham; leucine 311 mC/mMole, isoleucine 308 mC/mMole and phenylalanine 459 mC/mMole), both dissolved in 20 µl insect Ringer were injected abdominally, first cycloheximide and immediately thereafter the precursors. For each point in figs. 1–3 the flight muscles of 4 locusts (2 females, 2 males) were pooled. Mitochondria were prepared according to the procedure of Klingenberg et al. [10], with the exception that the isolation medium contained the unlabelled precursor amino acids in 10 mM concentrations. The supernatant after centrifugating the homogenate 30 min at 20,000 × *g* was called the 20,000 × *g* supernatant and contained microsomes and cell sap. Mitochondria were labelled *in vitro* by incubation in a medium described by Bronsert et al. [2]. The preparation of insoluble mitochondrial protein from the mitochondria [5], the procedure for the determination of specific radioactivity in the protein fractions [4], and the conditions for electrophoresis and for determination of radioactivity in the pherograms [5] have been described.

3. Results

As shown in fig. 1 the inhibition of protein synthesis is maximal at 20 µg of cycloheximide per locust and remains constant at higher levels. At maximal inhibition the radioactivity still incorporated is for the proteins of the 20,000 × *g* supernatant (S) less than 3% of the control without cycloheximide, but for the whole mitochondrial protein (M) 15–20%.

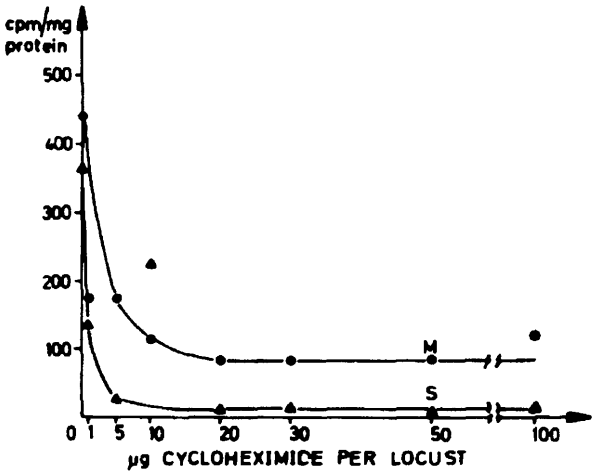

Fig. 1. Incorporation *in vivo* of 14-C-phenylalanine (0.2 μC per locust) into protein of whole mitochondria (M, ●—●) and 20,000 X*g* supernatant (S, ▲—▲) in the presence of different amounts of cycloheximide. The locusts were killed 30 min after the injection of the precursor.

In the control experiment without cycloheximide (fig. 2a) the rate of amino acid incorporation is nearly linear for the first 60 min, and then declines, probably due to exhaustion of the precursor. Mitochondrial (●) and supernatant (▲) proteins are labelled to the same extent. In the presence of cycloheximide (fig. 2b) labelling of the supernatant proteins is negligible while incorporation into the whole mitochondrial proteins still proceeds during the first 30 min in a linear rate and has stopped at 60 min. The incorporation rate within the first 30 min is about 15% of the control experiment. Observations have shown that 30 min after the injection of 50 μg of cycloheximide per locust the protein synthesis in the microsomes begins again. Therefore the same amounts were reinjected every 30 min.

After incorporation of amino acids *in vivo* in the presence of cycloheximide, the insoluble mitochondrial proteins (80% of the whole mitochondrial protein) are labelled to a much higher extent than the soluble mitochondrial proteins, as shown in table 1. The same is true for isolated mitochondria labelled by incubation *in vitro*.

By gel electrophoresis in a strong acid medium [5] the insoluble mitochondrial proteins are resolved into approximately 20 bands (fig. 3). The *in vivo* incorporation of the 14-C-amino acids into the bands is approximately proportional to the amido black staining. *In vivo* in the presence of cycloheximide radioactivity is incorporated only into bands 2, 4, 5b, 6, 8 and 9. Band 4 is mainly labelled, containing 25% of the total radioactivity. When mitochondria are incubated *in vitro*, 14-C-amino acids are incorporated into the same bands of electrophoretically separated insoluble mito-

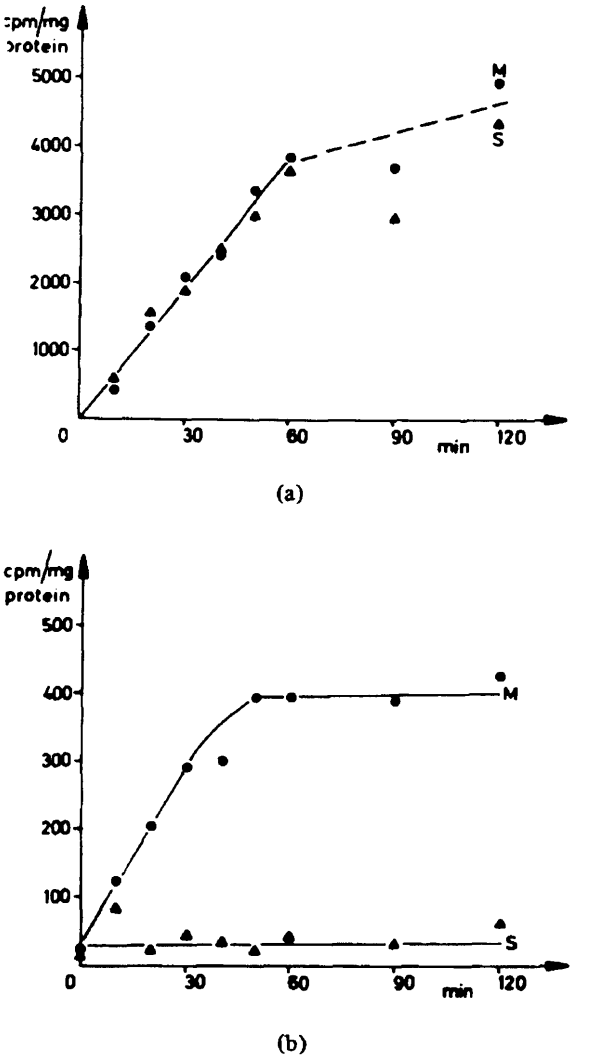

Fig. 2. Time course of incorporation *in vivo* of 14-C-leucine plus 14-C-isoleucine plus 14-C-phenylalanine (0.2 μC each per locust) into protein of whole mitochondria (M, ●—●) and 20,000 X*g* supernatant (S, ▲—▲). (a) Without cycloheximide. (b) After the injection of 50 μg cycloheximide per locust. 50 μg cycloheximide was reinjected every 30 min.

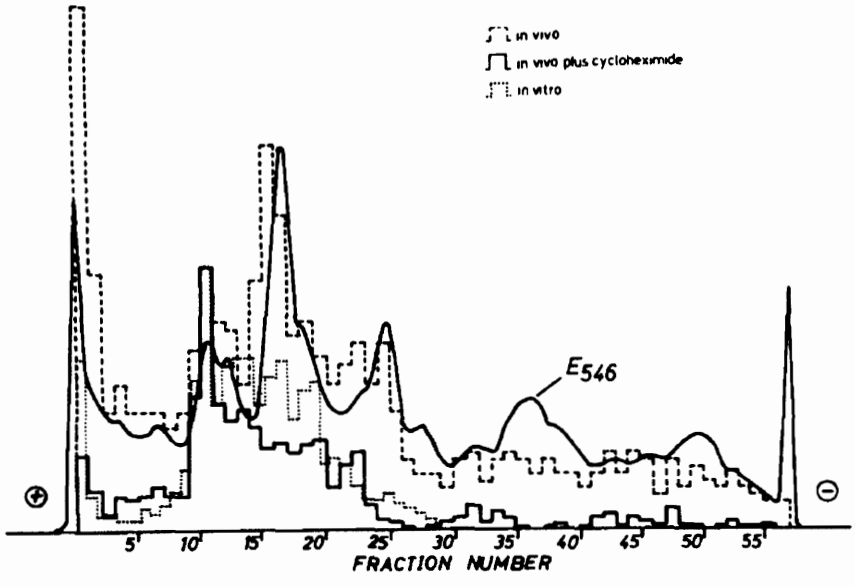

Fig. 3a. Densitogram of amido black stained insoluble mitochondrial protein after gel electrophoresis (smooth line), and distribution of incorporated radioactivity over the pherograms (edged lines). – – – *in vivo*, —— *in vivo* plus cycloheximide, · · · · · *in vitro*. For labelling conditions see table 1. Radioactivity in band 4 (fraction 11) was taken as equal.

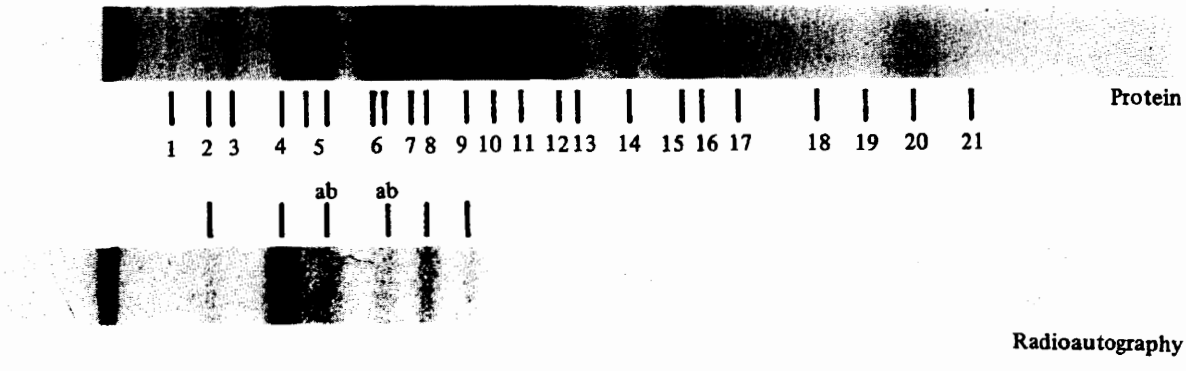

Fig. 3b. Electrophoretic pattern (same expansion as in fig. 3a) of insoluble mitochondrial protein and radioautography from the pherogram. The insoluble mitochondrial protein was labelled *in vivo* in the presence of cycloheximide, as described in table 1.

Table 1
Specific radioactivities of submitochondrial fractions

Mitochondrial fractions	Mitochondrial labelling (cpm per mg protein)		
	in vivo [a]	*in vivo* with cycloheximide [b]	*in vitro* [c]
Insoluble protein	9200	3640	11 000
Soluble protein	8500	540	900

[a] 1 hr with 0.4 μC leucine, 0.4 μC isoleucine, and 0.4 μC phenylalanine per locust.
[b] 1 hr in the presence of 50 μg cycloheximide per locust (50 μg cycloheximide was reinjected after 30 min) with 2 μC leucine, 2 μC isoleucine, and 2 μC phenylalanine per locust.
[c] 30 min with 1 μC leucine, 1 μC isoleucine, and 1 μC phenylalanine per ml.

chondrial protein as *in vivo* in the presence of cycloheximide. However, the proportions are different, band 4 containing a relatively lower label.

4. Discussion

Since microsomal protein synthesis was completely inhibited by cycloheximide from the beginning, these experiments demonstrate *in vivo* the existence of a mitochondrial amino acid incorporating system similar to that already established with isolated mitochondria *in vitro*. Remarkably this system continues to operate in the presence of cycloheximide for at least 30 min after the rest of cellular protein synthesis has stopped.

The principally labelled electrophoretic protein fraction *in vivo* in the presence of cycloheximide as well as *in vitro* is band 4, one of the smaller slowly moving components. In experiments with mitochondria isolated from *Neurospora crassa* [5] the predominantly labelled protein fraction shows characteristics similar to band 4 of the locust flight muscle mitochondria. Interestingly enough, this fraction from *Neurospora* wild type mitochondria is nearly absent in mitochondria of the cytoplasmic mutant mi-1 (poky) [5]. This "band 4 protein" may therefore play an important role in the biogenesis of mitochondria.

Acknowledgements

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