CYCLOHEXIMIDE-RESISTANT AMINO ACID INCORPORATION INTO RAT LIVER MITOCHONDRIAL PROTEINS *IN VIVO*

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1. Introduction

Isolated rat liver mitochondria incorporate amino acids into a heterogeneous insoluble membranous fraction, representing less than 10% of the total mitochondrial protein [1]. The remaining mitochondrial proteins appear to be synthesized extramitochondrially on cycloheximide-sensitive ribosomes and transferred into the mitochondrial structure in a subsequent step [2]. The process of mitochondrial biogenesis has been approached in the present study by taking advantage of the observation that cycloheximide does not inhibit amino acid incorporation into protein by isolated mitochondria *in vitro* [3, 4], while inhibiting protein synthesis in mammalian ribosomes [5]. This selective inhibition by cycloheximide makes it possible to differentiate *in vivo* the site of synthesis of various mitochondrial proteins. Thus, any *in vivo* labeling of mitochondrial proteins observed in animals treated with sufficient cycloheximide to block completely protein synthesis in the microsomes must represent protein synthesis by the mitochondria. Studies *in vivo* also avoid many of the uncertainties of experiments *in vitro* in which conditions may not be adequate for maximum labeling of some proteins or protein complexes.

In the present study, rats received by intravenous injection either ^{3}H-leucine or ^{3}H-leucine plus cycloheximide. The results suggest that the amino acid incorporation into insoluble membranous protein observed *in vitro* also occurs *in vivo* and that a small percentage (15 to 20) of the proteins associated with a purified preparation of cytochrome oxidase may be synthesized by the cycloheximide-insensitive system of the mitochondria.

2. Methods

Adult male rats weighing approximately 150 g received 40 or 50 μCi of 4,5-^{3}H-L-leucine by intravenous injection and were killed either two or thirty minutes later. Liver mitochondria were prepared in 0.25 M sucrose containing 0.01 M tris, pH 7.6, as previously described [6]. Mitochondria obtained from one rat liver were extracted with acetic acid, to yield a protein fraction soluble in acid plus a membranous residue [7]. Alternately, mitochondria obtained from three rat livers were pooled and fractionated with Triton X-114 to yield a soluble preparation of cytochrome oxidase [8, 9]. After the initial centrifugation in each preparation, the Triton X-114 soluble fraction was saved in addition to the hard-packed material at the bottom of the centrifuge tube which was mechanically separated from the green sliding pellet containing cytochrome oxidase [1].

Cycloheximide was dissolved in 0.9% aqueous saline and injected intraperitoneally to the rats (5 mg/100 g body weight) twenty minutes prior to the ^{3}H-leucine.

Radioactive proteins were prepared for counting by previously described methods [3].

3. Results and discussions

As seen in table 1, at two minutes after injection

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Table 1
Distribution of radioactivity *in vivo* in submitochondrial fractions obtained after acetic acid treatment.

Fraction	2 min		30 min			
	cpm/mg	Ratio [a]	cpm/mg	+ Cyclo-heximide	Ratio [a]	% Control
Mitochondria	87.7	1.00	126	19.4	1.00	15.3
Acid soluble	51.0	0.58	114	5.53	0.29	4.8
Acid residue	213	2.43	137	41.9	2.15	30.6
Microsomes	–	–	1180	30.8	–	2.6

Liver mitochondria obtained from rats which had received 40 μCi of ^{3}H-leucine were fractionated with acetic acid. Each value is the average of 6 experimental animals.

[a] The specific activity of each fraction is compared to that of the unfractionated mitochondria.

of ^{3}H-leucine the specific activity of the fraction containing the insoluble membranous proteins (those proteins not extracted with acetic acid) was 2.4 times that of the whole mitochondria, while thirty minutes after the injection of ^{3}H-leucine the specific activity of all the submitochondrial fractions was identical. This result suggests that the proteins of this fraction are labeled most rapidly in the mitochondria and hence may be synthesized within the mitochondria assuming an immediate distribution of the radioactive amino acid throughout the cell after injection.

The results obtained in animals treated with cycloheximide support this conclusion. The amount of cycloheximide used in these experiments was sufficient to block protein synthesis 98% in the microsomes; however, only an 85% inhibition of labeling was observed in the intact mitochondria. The mitochondrial proteins extracted with dilute acetic acid were inhibited 95% while the membrane fraction was only inhibited 70% by the cycloheximide. It thus appears that approximately 30% of the proteins in this fraction may be synthesized by the cycloheximide-insensitive system of the mitochondria.

This result was anticipated since our previous study [1] had shown that isolated rat liver mitochondria almost quantitatively incorporate amino acids *in vitro* into the proteins of the residue after acetic acid extraction. After gel electrophoresis, two of the slowest moving bands plus the material at the origin of the gel contained significant radioactive labeling. These bands represented approximately 25 to 30% of the protein of the fraction. It thus appears that the radioactive labeling of mitochondrial proteins observed *in vitro* in isolated mitochondria is identical to that occurring *in vivo*, when protein synthesis on the microsomes is blocked with cycloheximide.

Similar results have been obtained *in vivo* with either yeast or *Neurospora*. Approximately, 15% of the soluble membrane proteins of yeast mitochondria were labeled *in vivo* in the presence of cycloheximide [10]. A slightly higher percentage of the membrane proteins of *Neurospora* mitochondria were synthesized by the cycloheximide-insensitive system [11]. In addition, the proteins of *Neurospora* mitochondria labeled *in vivo* in the presence of cycloheximide were shown by gel electrophoresis to be identical to the proteins labeled *in vitro* by these mitochondria [12].

It was also of some interest to study the *in vivo* labeling of cytochrome oxidase in the presence of cycloheximide. A soluble preparation of cytochrome oxidase was labeled in animals treated with cycloheximide. Hence, it appears that the majority of the proteins of this cytochrome oxidase preparation is not synthesized within the mitochondria by the cycloheximide-resistant system of protein synthesis. Schiefer [13] also observed that cycloheximide inhibited the *in vivo* labeling of a membranous preparation of cytochrome oxidase.

Similarly, only a slight labeling of a soluble cytochrome oxidase was observed when isolated rat liver mitochondria were incubated with ^{14}C-leucine [1]. The radioactivity present in the purified cytochrome oxidase was not associated with the two major bands observed after gel electrophoresis, although significant counts were present in the slow moving band also observed. These results plus those of the present study

Table 2
Distribution of radioactivity *in vivo* into cytochrome oxidase.

Fraction	cpm/mg Protein			
	Control	+ Cyclo-heximide	Ratio [a]	% Control
Mitochondria	327	23	1.00	7
Triton soluble	340	18	0.78	5.3
Cytochrome oxidase (soluble)	200	41	1.78	20.4
"Button"	280	57	2.48	20.3
Microsomes	2110	39	–	1.8

Mitochondria obtained from 3 rat livers were pooled prior to fractionation with Triton X-114 and Triton X-100 to yield cytochrome oxidase. The animals were treated with 50 μCi of ^{3}H-leucine 30 min prior to sacrifice. Each value is the average of 3 experiments.

[a] The specific activity of each fraction is compared to that of the unfractioned mitochondria.

suggest that the two major proteins present in cytochrome oxidase [14] are not synthesized within the mitochondria and that the small, but persistent, radioactive labeling of the purified cytochrome oxidase either *in vitro* or *in vivo* in the presence of cycloheximide may be due to a minor protein associated with cytochrome oxidase in the inner membrane. Perhaps, this protein may be involved in the formation of a functional cytochrome oxidase because of this association. In this context, Chan and Charalampous [15] have recently concluded that the biogenesis of cytochrome oxidase in yeast involves proteins synthesized both by the cytoplasmic and the mitochondrial systems for protein synthesis.

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