SYNTHESIS OF MITOCHONDRIAL PROTEINS IN ISOLATED RAT HEPATOCYTES

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1. Introduction

Mitochondrial synthesis has been extensively studied in lower eucaryotic cells [1–3]. Comparatively little, however, is known about this process in intact higher eucaryotic cells, and the bulk of this information is restricted to rapidly growing, often poorly differentiated, cells in culture [1,4–7]. Until recently, few systems were available with which to study the biosynthesis of mitochondrial membranes in the intact, well differentiated, normal mammalian cell. Such a system is required if we are to understand the nucleo–cytoplasmic interactions in normal higher eucaryotic cells, or the possible influence of hormones and growth factors [8,9] on biosynthesis. As such an experimental system, the isolated hepatocyte offers the following advantages:

(1) Much is already known about the synthesis of rat liver mitochondrial membranes, based primarily on in vitro labeling of isolated mitochondria [1,10,11];
(2) Hepatocytes can be maintained in suspension for several hours, and can be manipulated with substrates and inhibitors;
(3) Large quantities of cells are available for subcellular fractionation;
(4) Nucleo–cytoplasmic relationships can be studied;
(5) The cells retain full sensitivity to hormones and other growth factors [9].

In contrast to other mammalian cell types which have been studied in tissue culture [4–7], growth of freshly isolated hepatocytes is slow, or non-existent [9]. Mitochondrial synthesis could, thus, also prove so slow as to make impractical biosynthetic studies with the intact hepatocyte. Such studies were attempted [12] using homogenates of isolated hepatocytes, but the labeling found in immunoprecipitated peptides corresponding electrophoretically to subunits of cytochrome oxidase, was very low, almost negligible. Furthermore, the mitochondrial origin of these peptides could not be rigidly demonstrated since they were not detected in immunoprecipitates of isolated mitochondria.

Here we have re-investigated the possible use of isolated hepatocytes as a model system for studying mitochondrial biosynthesis in well-differentiated mammalian cells. Using methods developed for the small scale preparation of mitochondria from isolated hepatocytes [13], we were able to analyze the mitochondrial peptides translated on mitochondrial and cytoplasmic ribosomes. A minimum of 7–8 mitochondrially-translated peptides can be detected, 4 of which make up 80–90% of the total label. These peptides are synthesized synchronously, and are stable after a 3–4 h pulse.

2. Methods

Rat hepatocytes were isolated from 180 g male Sprague-Dawley rats as in [14], with the modifications in [13]. Hepatocytes, prepared according to this method, were 85–95% viable as determined by trypan blue exclusion and NADH permeability.

Isolated hepatocytes (10 mg protein/ml, 3×10^6 cells/ml) were labeled in 10 ml of media [14] containing 17.5% (v/v) heat-inactivated horse serum, 0.6% glucose, heparin (7 units/ml) and 1.52 mM of an

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amino acid mixture [14]. Labeling was started by the addition of either [^{35}S]methionine or [^{3}H]leucine. The amount of isotope used in each experiment is given in the figure legends. The cells were incubated up to 4 h at 37°C in 50 ml spinner-flasks as in [15]. Incorporation of radioactive amino acid was stopped by addition of cold amino acid (40 mM final conc.). Labeled cells (100 mg protein) were washed and resuspended in 20 ml 0.25 M sucrose containing 0.5 mM cycloheximide and 5 mM chloramphenicol, and mitochondria were isolated after a brief sonication, as in [13]. Submitochondrial particles were prepared by the method in [16]. Labeled membranes were solubilized in SDS and electrophoresed on 12% or 15% acrylamide slab gels using the buffer system in [17]. The gels were either sliced (1 mm slices) for scintillation counting, or analyzed by autoradiography or fluorography using Kodak X-Omat film.

3. Results

Hepatocytes incubated in vitro with [^{35}S]methionine efficiently incorporate isotope into mitochondrial protein. In the absence of inhibitor, submitochondrial particles are labeled with spec. act. 4–5 X 10^5 cpm/mg protein after 4 h of incubation. About 90% of this labeling is prevented by cycloheximide.

Figure 1 shows the time course for labeling of intact cells, mitochondrial and submitochondrial particles in the presence of [^{35}S]methionine and cycloheximide. Enrichment of specific radioactivity during preparation of submitochondrial particles from mitochondria is identical to the enrichment of cytochromes, indicating that nearly all of the label in mitochondria is located in the inner membrane.

Labeling is linear for the first 2 h of continuous incubation in the presence of [^{35}S]methionine and cycloheximide (fig.1). The decrease after 2 h is not, however, due to inhibition of mitochondrial protein synthesis. This could be shown by giving the cells a 1 h pulse of [^{35}S]methionine in the presence of cycloheximide during hour 1 and 4 of incubation. In 3 separate experiments, the incorporation of [^{35}S]methionine was found not to be inhibited after preincubation, even if preincubation was carried out in the presence of cycloheximide. We conclude that the non-linear labeling shown in fig.1 is due either to depletion of [^{35}S]methionine via metabolism [18], or to dilution in a continuously expanding pool of unlabeled methionine.

Figure 2 shows the gel patterns of mitochondrial

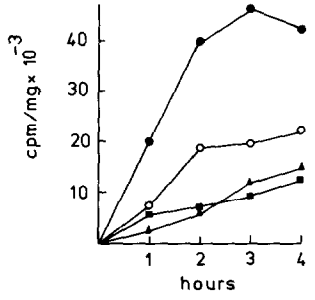

Fig.1. Time course of synthesis of mitochondrial proteins in isolated hepatocytes incubated in the presence of cycloheximide. Rat hepatocytes (10 mg protein/ml) were incubated with [^{35}S]methionine (15.8 μCi/ml) in the presence of 0.5 mM cycloheximide. Samples were removed at the indicated time intervals, and radioactivity was measured in: submitochondrial particles (●-●); mitochondria (○-○); postmitochondrial supernatant (▲-▲); intact hepatocytes (■).

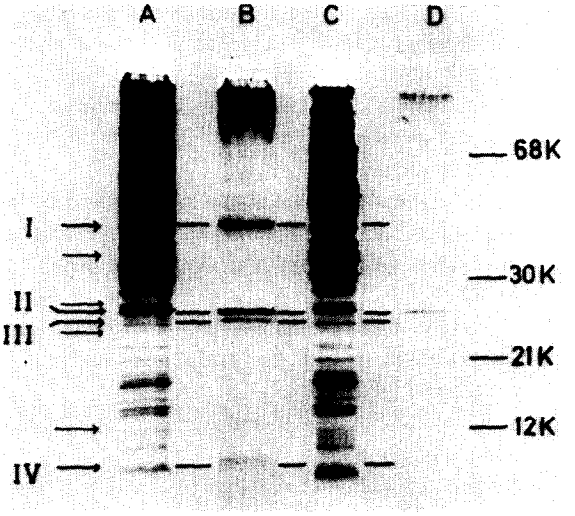

Fig.2. Autoradiographic analysis of mitochondrial proteins synthesized by hepatocytes incubated in the presence and absence of inhibitors. Hepatocytes were labeled for 4 h with [^{35}S]methionine (18 μCi/ml) after which submitochondrial particles were prepared and electrophoresed as in section 2. (A) No inhibitor; (B) 0.5 mM cycloheximide; (C) 0.05 mM chloramphenicol; (D) 0.5 mM cycloheximide plus 0.05 chloramphenicol.

inner membranes prepared from hepatocytes labeled in the presence and absence of inhibitors. Four mitochondrial peptides are relatively strongly labeled in the presence of cycloheximide (fig.2B). Together they make up 80–90% of all the radioactivity. Their apparent molecular weights are: I (45 000); II (26 000); III (24 000); IV (⩽ 10 000). In addition, 3–5 weakly-labeled bands can be detected in different experiments. Although these bands can not be seen on the particular autoradiograph shown in fig.2, their positions on the gels are marked with arrows. Labeling of all the above peptides is inhibited when cells are incubated in the presence of chloramphenicol (fig.2C,D). Chloramphenicol inhibition is not complete, however, due to the low concentration (50 μM) used in our experiments. The selection of this concentration of chloramphenicol was based upon titration experiments which showed that higher concentrations also inhibit cytoplasmic protein synthesis, thus decreasing the specificity of its inhibition.

Experiments similar to those in fig.2 were also carried out using [^{3}H]leucine as label. Bands I–IV could easily be discerned in the inner membranes isolated from cells labeled in the presence of cycloheximide.

The time course of labeling of mitochondrially-made peptides is shown in fig.3. All of the peptides appear to be synthesized synchronously, and no evi-

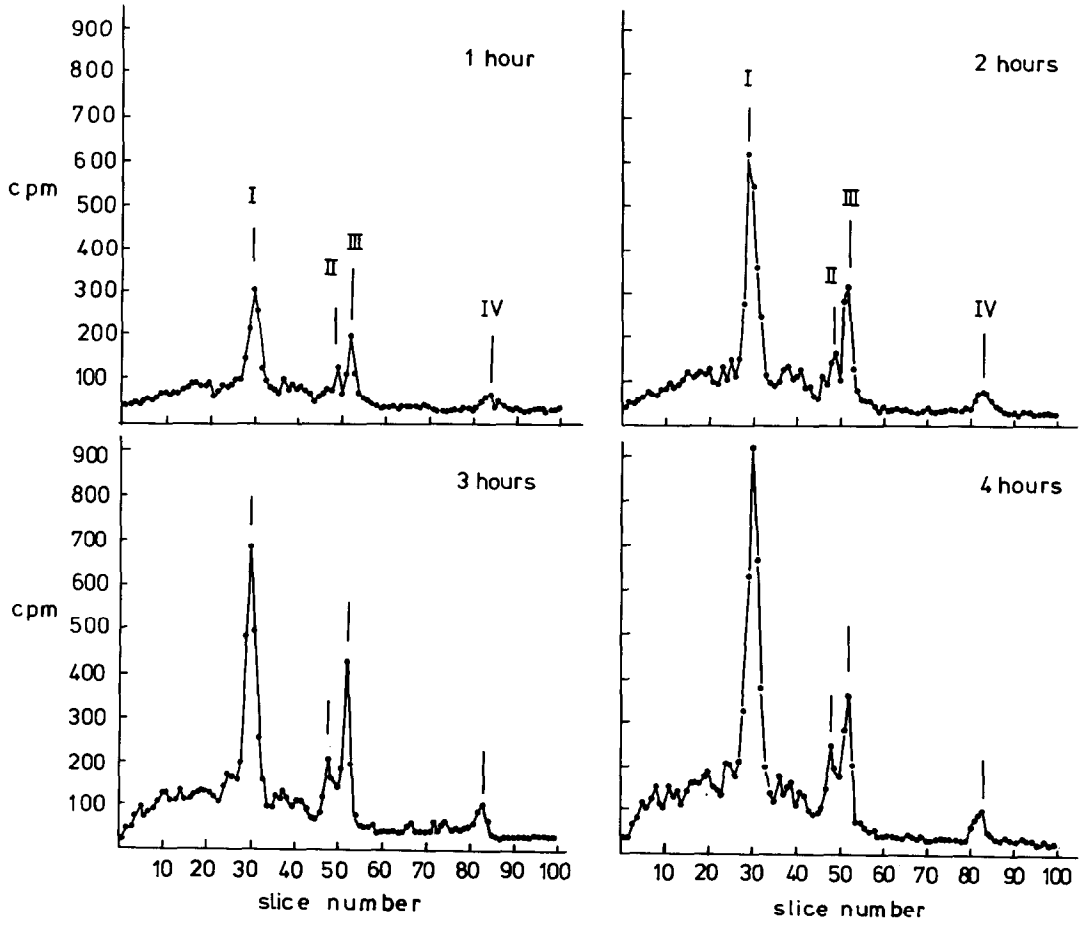

Fig.3. Electrophoretic analysis of the peptides synthesized by mitochondria after different times of incubation. Hepatocytes were incubated as in fig.1. Submitochondrial particles were prepared at the time interval indicated and electrophoresed on SDS polyacrylamide slab gels.

dence exists for product–precursor relationships. Pulse-chase experiments were also conducted in which hepatocytes were incubated for 1 h in the presence of cycloheximide, and then chased with cold methionine (2 mM) for 2–3 h in the presence of cycloheximide. Incorporation of [^{35}S]methionine into total cellular protein and inner membrane protein was inhibited immediately upon addition of the methionine chase, and the specific radioactivity remained constant during the 3 h chase. Electrophoretic analysis of the mitochondrially-translated peptides indicated that these products were stable after 3 h chase, and that the relative distribution of radioactivity between the various peaks did not differ significantly from that shown in fig.3. These findings again provide no support for an interconversion between the peptides [7]. They also indicate that breakdown of newly synthesized proteins is negligible under the conditions of our experiments. In the latter respect, the results from intact hepatocytes differ clearly from those obtained with isolated liver mitochondria labeled in vitro [19].

4. Discussion

This study clearly shows that mitochondrial protein synthesis can be measured in isolated rat hepatocytes, at least during the first 4 h incubation. In the absence of inhibitors, inner membrane preparations can be highly labeled (∿ 500 000 cpm/mg protein). Cycloheximide inhibits incorporation by 90–95%, and chloramphenicol inhibits the remaining incorporation. A maximum of 7–9 peptides are labeled in the presence of cycloheximide, and 4 of these contain 80–90% of the radioactivity. Since 12 [6] or 19 [20] mitochondrially-translated peptides have been reported in rapidly growing established human cell lines, the smaller number detected in hepatocytes could reflect the slower growth rate of the cell and, subsequently, a slower rate of mitochondrial protein synthesis. Though remote, the possibility can not be entirely excluded that these differences are due to species differences or to phenotypic changes in established cell lines. In this respect it should be noted that only 9 mitochondrially-translated peptides have been described in yeast [2].

The conditions of labeling described here, combined with methods developed for the rapid, small-scale preparation of mitochondria from isolated hepatocytes [13], provide us with a unique experimental system to study mitochondrial membrane synthesis in highly differentiated mammalian cells. Experiments are currently underway to identify the mitochondrially-translated peptides using antibodies directed against inner membrane protein.

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