BIOGENESIS OF THE OUTER MITOCHONDRIAL MEMBRANE IN ISOLATED RAT HEPATOCYTES

Pär GELLERFORS and Monica LINDÉN
Department of Biochemistry, Arrhenius Laboratory, University of Stockholm, S-106 91 Stockholm, Sweden

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1. Introduction

The outer membrane from rat liver mitochondria was first isolated in 1966 [1,2]. Since then very little attention has been paid to the synthesis and assembly of the outer membrane. Today synthesis and assembly of the outer membrane is becoming increasingly important, particularly in view of its possible role in import of cytoplasmically made matrix proteins [3]. For example, to explain the specific recognition of imported proteins by the mitochondrion, several investigators have proposed the presence of receptor proteins in the outer membrane [3–5]. If such a receptor exists, however, one must solve the paradox as to how the receptor protein recognizes the mitochondrial membrane. One obvious possibility is that such protein might be coded for by mitochondrial DNA. This is also suggested by earlier experiments, which showed a small but significant labeling of the outer membrane in in vitro labeled mitochondria [6,7].

Here, we have studied biosynthesis of the outer mitochondrial membrane in isolated rat hepatocytes. Our results show that all of the peptides of the outer membrane, including the pore protein [8], are translated on cytoplasmic ribosomes.

2. Methods

Hepatocytes were isolated from male Sprague-Dawley rats (180 g body wt) as in [9]. Isolated cells were labeled in the presence of [^{35}S]methionine (30 μCi/ml) and various inhibitors of protein synthesis [10]. After labeling, cells were washed and the mitochondria were isolated as in [11]. The outer mitochondrial membrane was subsequently isolated from ~40 mg labeled mitochondria as in [12], using a SW-56 rotor for the small scale preparation. Purified outer membrane (~0.4 mg) was obtained from 40 mg mitochondria. The outer membrane preparation was analyzed by SDS–polyacrylamide gel electrophoresis [13] and fluorography [14]. Pore protein was isolated from rat liver mitochondrial outer membranes according to [8]. Cytochrome b_5 isolated from rat liver according to [15], was generously supplied by Dr Åke Elhammer. Protein was determined according to [16]. Cytochrome oxidase [17] and monoamine oxidase [18] activities were determined as described.

3. Results and discussion

3.1. *Characterization of the outer mitochondrial membrane prepared from isolated rat hepatocytes*

The outer mitochondrial membrane was isolated starting from 150–200 mg hepatocyte protein (see section 2). The small scale procedure gave an outer mitochondrial membrane preparation, which was highly enriched in monoamine oxidase activity (~20-fold over mitochondria) and contained <3% contaminating inner mitochondrial membrane as judged by the specific cytochrome oxidase activity (table 1). The outer membrane preparation revealed 6–7 heavily-stained

Table 1
Characterization of the outer mitochondrial membrane preparation isolated from rat hepatocytes

Fraction	Cytochrome oxidase (nmol/min mg)	Monoamine oxidase (nmol/min mg)
Mitochondria	2430	6.1
Crude inner membrane	4080	5.7
Outer membrane	137	111

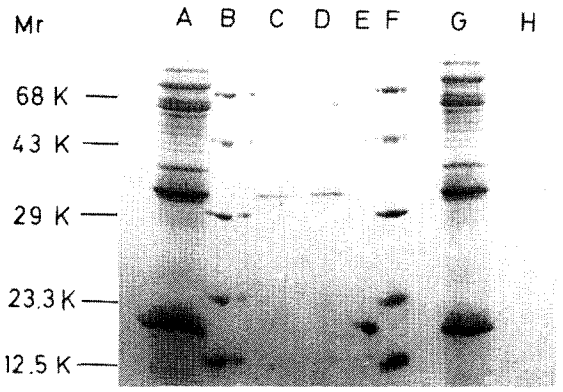

Fig.1. SDS–polyacrylamide gel electrophoresis of the outer membrane from rat liver mitochondria. Rat liver outer mitochondrial membranes were purified according to [12]. Pore protein was subsequently solubilized from the outer mitochondrial membrane in NaCl–Triton X-100 and isolated by differential centrifugation and sucrose gradient centrifugation as in [8]. Cytochrome b_5 was isolated as in [15]. All fractions were electrophoresed on a 12.5% SDS–polyacrylamide gel in a Tris–glycin buffer system as in [13]. Outer mitochondrial membranes (A,G); pore protein (C,H); NaCl–Triton X-100-soluble fraction before sucrose gradient centrifugation (D). Cytochrome b_5 (E). Standard proteins for M_r determinations were: albumin (68 000); ovalbumin (43 000); carbonic anhydrase (29 000); trypsin inhibitor (23 300); cytochrome *c* (12 500) (B,F).

polypeptide bands on SDS–polyacrylamide gel electrophoresis as well as several minor bands (fig.1 A,G). The identities of two of the heavily-stained polypeptide bands were determined by coelectrophoresis of purified pore protein (C,H) [8], purified cytochrome b_5 (E), and intact outer mitochondrial membranes (A,G). The app. M_r values of these proteins were 31 000 and 15 000, respectively. These two proteins represent ~20–30% of the proteins of the outer membrane as judged by Coomassie blue staining (A).

3.2. *Biogenesis of the outer mitochondrial membrane*

Hepatocytes were labeled with [^{35}S]methionine in the absence or presence of inhibitors of protein synthesis. After labeling, the outer membrane was isolated and analyzed.

The outer mitochondrial membrane, when isolated from cells incubated either in the absence or in the presence of chloramphenicol, showed approximately the same specific radioactivity (table 2). However, when labeled in the presence of cycloheximide the specific radioactivity was reduced to 12% of that in the control (no inhibitor), in agreement with [6,7].

Table 2
Radioactivity found in the outer mitochondrial membrane when isolated from hepatocytes labeled under different conditions

Condition	cpm/mg ($\times 10^{-3}$)
No inhibitor	487
Chloramphenicol 0.05 mM	467
Cycloheximide 0.05 mM	59

Hepatocytes were labeled with [^{35}S]methionine (30 μCi/ml) in the absence or presence of cycloheximide or chloramphenicol, and the outer membrane was subsequently isolated and analyzed for specific radioactivity

However, to analyze the products found in the outer membrane more accurately, under different labeling conditions, it was subjected to SDS–polyacrylamide gel electrophoresis and fluorography. Fig.2 shows the

Fig.2. Biosynthesis of outer and inner mitochondrial membrane proteins, in isolated rat hepatocytes in the presence or absence of protein synthesis inhibitors. Isolated rat hepatocytes were labeled with [^{35}S]methionine in the absence or presence of 0.5 mM cycloheximide or 0.05 mM chloramphenicol. The outer and inner membranes were isolated and analyzed by SDS–polyacrylamide gel electrophoresis (12.5%) and fluorography. Inner mitochondrial membrane labeled in the absence (A) or in the presence of cycloheximide (B) or chloramphenicol (C). Outer mitochondrial membrane labeled in the absence (D) or in the presence of cycloheximide (E) or chloramphenicol (F). Protein (100 μg) was applied to each lane.

labeling pattern of both the outer membrane (D–F) and, for comparison, the inner membrane (A–C). The labeling pattern of the outer membrane in the absence of inhibitors revealed numerous labeled bands (D) migrating differently from the polypeptide bands of the inner membrane (A), showing the uniqueness of these two membranes. However, the number of [^{35}S]-methionine labeled bands associated with the outer membrane is in large excess of the number of Coomassie blue-stained bands (cf. fig.1 A). The reason for this is not known. One possible explanation is that cytoplasmically synthesized products have been trapped in the outer membrane during isolation. No major change in the labeling pattern is observed in the presence of chloramphenicol, although a slight increase in labeling of some components was found (F). However, in the presence of cycloheximide, no labeled bands are detected in the outer membrane (E), whereas 8–10 labeled bands are observed in the inner membrane (B) isolated from the same mitochondria. Occasionally, when highly labeled preparations were analyzed, faint bands could be observed in the outer membrane. These bands corresponded in mobility to the mitochondrial translational products found in the inner membrane and represent contaminating inner membrane proteins, in agreement with enzyme activity measurements (table 1). This also explains the low specific radioactivity found in the outer membrane in the presence of cycloheximide (table 2).

This study shows that all of the proteins of the outer mitochondrial membrane, including the pore protein [8] and cytochrome b_5, are synthesized on cytoplasmic ribosomes. No mitochondrially synthesized product was uniquely found in the outer membrane. We are now studying the process of biogenesis and assembly of the individual components of the outer membrane.

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