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Immunological detection of the mitochondrial F_1-ATPase α subunit in the matrix of rat liver peroxisomes

A protein involved in organelle biogenesis?

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Rat liver peroxisomes contain in their matrix the α-subunit of the mitochondrial F_1-ATPase complex. The identification of this protein in liver peroxisomes has been achieved by immunoelectron microscopy and subcellular fractionation. No β-subunit of the mitochondrial F_1-ATPase complex was detected in the peroxisomal fractions obtained in sucrose gradients or in Nycodenz pelletted peroxisomes. The consensus peroxisomal targeting sequence (Ala-Lys-Leu) is found at the carboxy terminus of the mature α-subunit from bovine heart and rat liver mitochondria. Due to the dual subcellular localization of the α-subunit and to the structural homologies that exist between this protein and molecular chaperones [(1990) Biol. Chem. 265, 7713–7716] it is suggested that the protein should perform another functional role(s) in both organelles, plus to its characteristic involvement in the regulation of mitochondrial ATPase activity.

α-F_1-ATPase; Molecular chaperone; Peroxisome; Mitochondria; Immunoelectron microscopy; Subcellular fractionation; Rat liver

1. INTRODUCTION

The mitochondrial F_0F_1-ATP synthase catalyzes the synthesis of ATP in oxidative phosphorylation at the expense of the proton electrochemical gradient generated by the respiratory chain. This inner membrane complex consists of two parts, the F_0, a proton channel embedded in the membrane and the F_1, a protein complex that protrudes from the membrane into the mitochondrial matrix and carries the functional domains for the synthesis and hydrolysis of ATP [1–3]. F_1-ATPase consists of five different subunits with the following proposed stoichiometry α_3, β_3, γ_1, δ_1, ϵ_1. The catalytic subunit of the F_1-complex is the β subunit. Until recently, the α subunit of the complex has received little attention, although its role in the rate of ATP synthesis and hydrolysis has been suggested [4–5].

ATPase activity has been identified in the membrane fraction of rat liver peroxisomes [6]. In contrast to mitochondrial F_1-ATPase, peroxisomal ATPase is a vacuolar-type ATPase (V-ATPase) [6]. V-ATPases, a new class of proton ATPases which are specifically associated with the endomembrane system of eukaryotes [1,7,8], function exclusively in the ATP hydrolytic direction generating a proton electrochemical gradient across the membranes of the vacuolar system [3]. V-ATPases are large multimeric complexes that contain, as the mitochondrial counterpart, an integral membrane domain and a hydrophilic catalytic complex. The V-type ATPase hydrophilic complex also contains 5 different subunits (A–E), but unlike the F_1-ATPase, the A and B subunits are considered to be the catalytic and regulatory subunits of complex, respectively [3].

In the present investigation we described, by means of immunoelectron microscopy and subcellular fractionation the existence of the mitochondrial α-F_1-ATPase regulatory subunit in rat liver peroxisomes. The existence of two different intracellular locations for this protein in addition to the striking structural homology of the α subunits from various organisms with molecular chaperones [9], led us to further suggest that the α-subunit could perform other functional role(s) in both organelles in addition to its involvement in mitochondrial oxidative phosphorylation.

2. MATERIALS AND METHODS

2.1. *F_1-ATPase purification and antibody production*

Rat liver mitochondrial F_1-ATPase was purified by chloroform extraction from purified inner mitochondrial membrane vesicles, as recently described in detail [10]. The purified F_1-ATPase complex (66 U/mg protein) had an estimated molecular weight by Sephadex G-200 gel filtration of 383 500 Da. Antibodies against rat liver mitochondria F_1-ATPase were raised in New-Zealand rabbits. The antibodies raised recognized by Western-blotting of mitochondrial proteins the major α and β subunits of the F_1-ATPase complex [10,11].

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2.2. *Electron microscopy*

Normal adult and fetal rat liver samples were fixed for 1 h with 1% glutaraldehyde in phosphate buffer saline (PBS) and 2% tannic acid [12,13] at room temperature. Samples were dehydrated with ethanol and embedded in Lowicryl K4M [14]. The initial steps of the dehydration process were carried out at 4°C, and after the 50% ethanol step, the samples were kept at −20°C. The resins were polymerized by irradiation with 360 nm UV light for 48 h at −20°C and 24 to 48 h at room temperature. Thin sections were adhered to nickel grids, which were floated on a drop of 1% ovalbumin in PBS for 15 min at 25°C. After the grids were transferred to a drop of a 1:100 dilution of rabbit anti-[rat liver mitochondrial F_1-ATPase] serum [10], they were incubated at 4°C for 12–16 h. The antibody was washed off with a stream of PBS, and the grids were treated with 1% ovalbumin and protein A-gold complexes in PBS for 1 h at 25°C, washed with water, dried, stained with saturated uranyl acetate, and finally washed with water. Colloidal gold particles of about 10-mn diameter were prepared [15]. The coupling of the colloidal gold to protein A from *Staphyloccocus aureus* was done as indicated by Roth [16]. The grids were examined in a JEOL 100B electron microscope.

2.3. *Subcellular fractionation of rat liver homogenates.*

1 g of adult rat livers were homogenized in 4 ml of ice-cold isolation medium (250 mM sucrose, 20 mM Tris-HCl, pH 7.4). The homogenates were centrifuged at 800 × *g* for 10 min. The resulting supernatants were fractionated on sucrose and Nycodenz-gradients.

2.3.1. Preformed sucrose gradients

Supernatants were layered on a 35 ml linear sucrose gradient (24–54%, w/w) in 20 mM Tris-HCl, pH 7.4, and centrifuged at 132400 × *g* in a VTi 50 vertical tube rotor in a L8-M Beckman ultracentrifuge for 70 min. 2 ml fractions were collected from the bottom of the tube. Catalase (EC 1.11.1.6) [17] and cytochrome *c* oxidase (EC 1.9.3.1) [18] activities were assayed in the homogenates and in the resulting fractions. Protein concentrations [19] were determined using crystalline bovine serum albumin as standard.

2.3.2. Self-generated gradients of Nycodenz

2 ml of the supernatants were layered on the top of 15 ml of a 27.6% Nycodenz isotonic solution (w/v) (5 mM Tris-HCl, pH 7.5, containing 3 mM KCl and 0.3 mM EDTA, refractive index of 1.3784 and a density of 1.15 g/ml) in 17 ml polyallomer thin-walled tubes. Tubes were centrifuged at 24000 rpm for 6 h in a Beckman L8-M centrifuge using a Sorvall AH627 rotor. 2 ml fractions were collected from the bottom of the tube. The compact pellet at the bottom of the tube was washed and resuspended in 1 ml of Nycodenz solution. Catalase, cytochrome *c* oxidase and acid phosphatase (EC 3.1.3.2) [20] activities and protein concentrations were assayed in the homogenates and in the resulting fractions.

2.4. *Other methods*

Fractionation of mitochondrial and peroxisomal proteins by polyacrylamide gel electrophoresis, electrophoretic transfer of the proteins to PVDF membranes, Western blotting with rabbit anti-[rat liver mitochondrial F_1-ATPase] serum and visualization of the immunoreactive proteins were carried out as recently reported in detail [10,11].

3. RESULTS

As expected, F_1-ATPase immunoreactivity in adult or fetal rat liver cells, revealed by the protein A-gold technique, was localized in the mitochondria (Fig. 1, Table I). Surprisingly, a high density of F_1-ATPase-immunoreactive material was localized in rat liver peroxisomes (Fig. 1; Table I). Several gold particles scattered throughout the cytoplasm were also visualized (Fig. 1; Table I), although the cytosolic immunoreactive material was only slightly above the background of the technique in adult liver thin sections (Table I). No

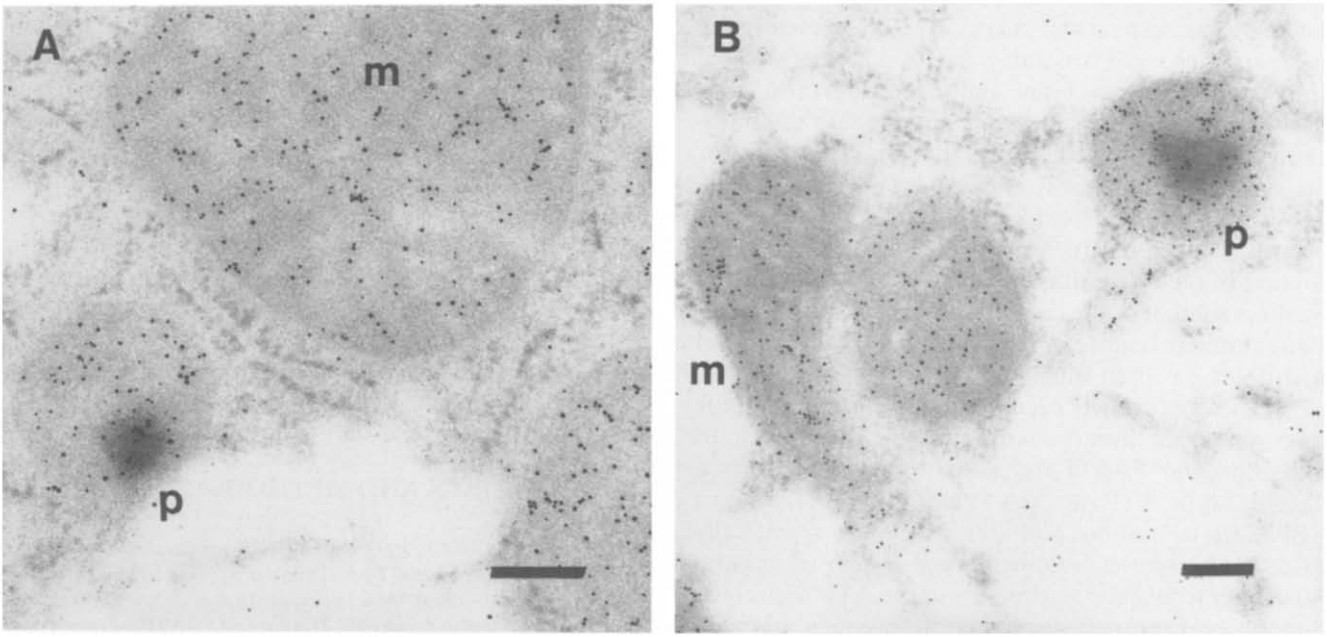

Fig. 1. F_1-ATPase immunoreactivity in adult (A) and fetal (B) rat liver cells. Adult and fetal rat liver samples were processed as described under Materials and Methods. Thin sections (A,B) were initially incubated with a 1:100 dilution of the anti-F_1-ATPase serum, washed and incubated with protein A-gold complexes (10 nm diameter) and stained with uranylacetate. Mitochondria (m) and peroxisomes (p) of rat liver cells stain heavily with the anti-F_1-ATPase serum. No labeling of subcellular structures were observed in thin sections incubated with preimmune serum. Bar, 500 nm.

Table I

Estimation of F_1-ATPase labeling density in rat liver cell compartments

Sample	Mitochondria	Peroxisome	Cytosol	Background
Fetal	318 ± 24	388 ± 20	12 ± 3	12 ± 1
Adult	354 ± 17	558 ± 40	22 ± 3	9 ± 1

To evaluate the specificity of labeling (gold particles/μm^2) in the various liver cell compartments, 9 and 16 electron micrographs of fetal and adult liver preparations, respectively, were taken at a magnification of 30 000. In the positive pictures (enlarged to a final magnification of 60 000) the number of gold particles per unit of area were counted. The results shown are means ± SE. The background was evaluated in 6 electron micrographs of fetal and adult liver thin sections incubated with preimmune rabbit serum

F_1-ATPase-specific immunoreactivity could be detected in any of the other liver cell compartments.

In order to characterize the molecular component(s) of the immunoreactive material found in liver peroxisomes, normal adult rat liver homogenates were fractionated in a linear sucrose gradient. The gradient allowed a good separation of both the mitochondrial and the peroxisomal fractions (Fig. 2A), as assessed by their respective enzyme marker activities; cytochrome *c* oxidase and catalase for mitochondria and peroxisomes, respectively. SDS-polyacrylamide gel electrophoresis fractionation of mitochondrial and peroxisomal proteins (fractions 5 and 15 of the gradient, respectively) and Western-blotting with rabbit anti-[rat liver mitochondrial F_1-ATPase] serum (Fig. 2B), revealed the expected existence of the α and β subunits of the F_1-ATPase complex in the mitochondrial fraction [10,21] and the existence of a single immunoreactive protein in the peroxisomal fraction with an apparent molecular weight similar to the α-subunit of the mitochondrial F_1 complex.

To verify the results obtained with sucrose gradients (Fig. 2B), rat liver homogenates were fractionated in self-generated gradients of Nycodenz. Nycodenz gradients allowed the recovery of a compact pellet of peroxisomes free from mitochondria and lysosomes, as assessed by the absence of cytochrome *c* oxidase and acid phosphatase activities, respectively (data not shown). In agreement with the results reported for sucrose gradients fractionated mitochondrial and peroxisomal proteins of the Nycodenz gradients (Fig. 2C) revealed the existence of the α and β subunits of the F_1-ATPase complex in the mitochondrial fraction, and the unique existence of an immunoreactive protein band in the peroxisomal pellet with an apparent molecular weight similar to the α-subunit of the mitochondrial F_1-ATPase complex.

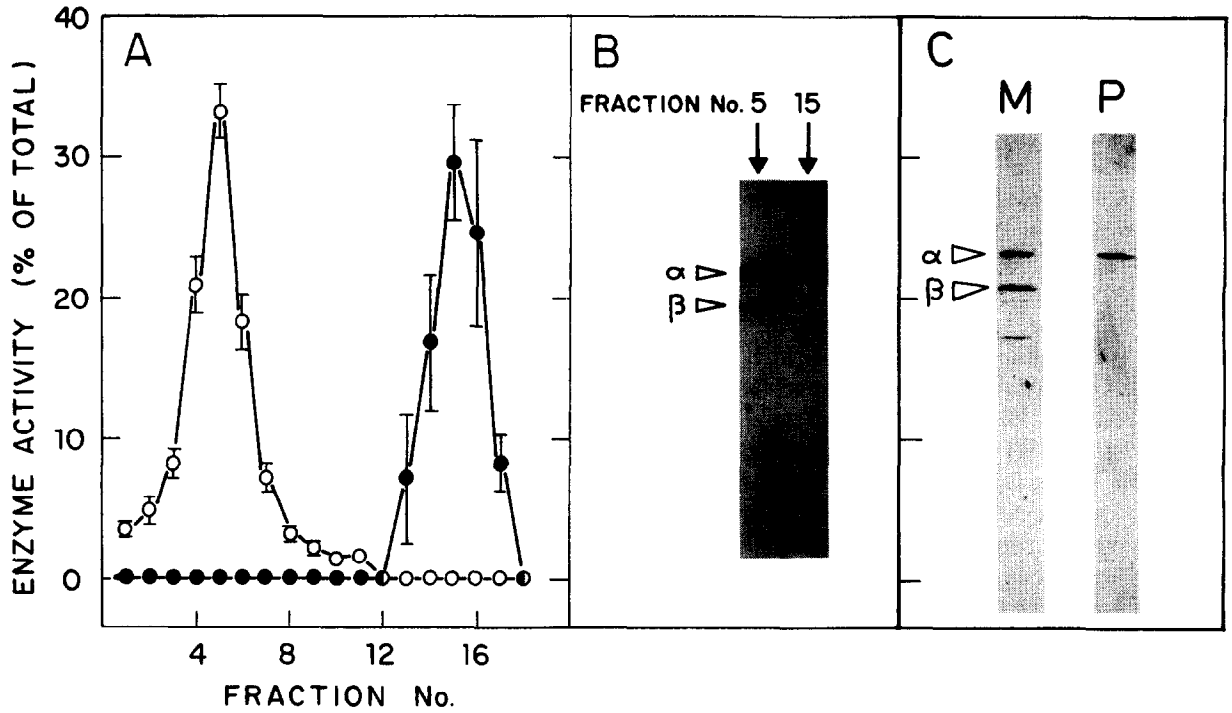

Fig. 2. Mitochondrial and peroxisomal isolation by sucrose and Nycodenz gradient fractionation of rat liver homogenates. Adult rat livers were homogenized and processed in linear sucrose gradients (A,B) or self-generated gradients of Nycodenz (C). (A) 2 ml fractions were collected from the bottom of the tube and assayed for cytochrome *c* oxidase (open circles) and catalase (closed circles). For each enzyme, the percent of the total activity loaded in the gradient and recovered in each fraction is represented. The results shown are means ± SE of 6 different experiments. (B) Autoradiography of a Western blot of 100 μg of mitochondrial (fraction 5) and peroxisomal (fraction 15) proteins obtained from the sucrose gradients probed with the anti-F_1-ATPase serum. (C) Western blot of 10 μg of mitochondrial (M) and peroxisomal (P) proteins obtained from the Nycodenz gradients.

4. DISCUSSION

The results reported in this paper show that rat liver peroxisomes contain in their matrix a protein with the same molecular weight and immunoreactivity towards the anti-F_1-ATPase antibody as mitochondrial F_1-ATPase α regulatory subunit. Western blot analyses are even more sensitive assays than the enzyme measurements reported herein. Thus, the finding that no F_1-ATPase β-subunit could be detected in sucrose or Nycodenz purified peroxisomes, as assessed by enzyme marker activities, further reinforce, by ruling out a possible mitochondrial contamination of the peroxisomal fractions, that the highly specific immunolabeling observed in the peroxisomal matrix is due to the presence of the α subunit protein in this organelle. The surprising result of a dual subcellular localization for a subunit of the oligomeric mitochondrial ATP synthase complex is not unique, since it has been recently documented that subunit *c* of the F_0 portion of the complex is accumulated in lysosomes [22]. Further, these results raise questions regarding the possible additional functional roles played by these proteins and on the mechanisms involved in the differential targeting of the proteins to the organelles.

The role of the α subunit in H^+-ATPases of the F-type, i.e., those present in plasma membrane of eubacteria and in the inner membrane of mitochondria and chloroplasts (for reviews see [1–3], is at present poorly understood. In V-type ATPases the regulatory subunit of the complex is the β subunit. This polypeptide is 5 kDa larger than the α subunit found in mitochondria and in the peroxisomes, and only shares a 25% overall amino acid identity with the latter [3]. The ATPase activity described in rat liver peroxisomes has been identified in the membrane fraction of the organelle [6], whereas the immunocytochemical localization of the α protein reveals that its main localization is in the peroxisomal matrix (Fig. 1). Therefore, it is reasonable to suggest for the α protein another yet unknown functional role not related to the peroxisomal ATPase. In such a situation, the α subunit of mitochondrial F_1-ATPase could perform the same functional role in the mitochondria, in addition to its characteristic involvement in oxidative phosphorylation. A bifunctional role for a mitochondrial protein has recently been documented [23]. In this regard, we have recently suggested [9], based on the rapid induction of the α protein in heat-shocked larvae of *Drosophila* and in the structural homologies detected among chaperones and the α subunits from various organisms, that the α protein could belong to the family of stress proteins hsp-60 [24,25]. If so, it could play, both in the mitochondria and in the peroxisomes, similar functional chaperone roles to those recently reported for mitochondrial hsp-60 [24,25], and hence, it would be involved in the biogenesis of both organelles.

Most mitochondrial proteins and the peroxisomal proteins are synthesized on cytosolic polysomes and further imported into their respective subcellular compartments (for reviews see [26–28]. The vast majority of inner membrane mitochondrial proteins carry a non-conserved amino-terminal presequence, the leader sequence or targeting signal, which is cleaved off during or after import into the mitochondria [26,27]. In contrast, peroxisomal proteins are synthesized in a mature form [28] with a non-proteolytically modifiable conserved targeting sequence, a tripeptide, mainly localized at the COOH terminus of the protein [29,30]. The present identification of the α subunit in rat liver peroxisomes necessarily implies the existence of a conserved peroxisomal targeting signal in its sequence. Analysis of the known mammalian mature α-subunit amino acid sequences of the F_1-ATPase from bovine heart [31] and rat liver [32] mitochondria revealed the existence of a consensus peroxisomal targeting sequence (Ala-Lys-Leu) [29] at positions 495–497 of its 509 amino acid sequence; suggesting because of the position of an analogous sequence found in human catalase, its classification as a Class II peroxisomal targeted protein [29].

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