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Identification of F_0 subunits in the rat liver mitochondrial F_0F_1-ATP synthase

F. Cretin [1], L.G. Baggetto [1], L. Denoroy [2] and C. Godinot [1]

[1] *LBTM-CNRS, Université Cl. Bernard de Lyon I, Villeurbanne (France) and* [2] *Centre de Microséquençage du CNRS Solaize (France)*

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In order to identify the subunits constituting the rat liver F_0F_1-ATP synthase, the complex prepared by selective extraction from the mitochondrial membranes with a detergent followed by purification on a sucrose gradient has been compared to that obtained by immunoprecipitation with an anti-F_1 serum. The subunits present in both preparations that are assumed to be authentic components of the complex have been identified. The results show that the total rat liver F_0F_1-ATP synthase contains at least 13 different proteins, seven of which can be attributed to F_0. The following F_0 subunits have been identified: the subunit *b* (migrating as a 24 kDa band in SDS-PAGE), the oligomycin-sensitivity-conferring protein (20 kDa), and F_6 (9 kDa) that have N-terminal sequences homologous to the beef-heart ones; the mtDNA encoded subunits 6 (20 kDa) and 8 (< 7 kDa) that can be synthesized in isolated mitochondria; an additional 20 kDa protein that could be equivalent to the beef heart subunit *d*.

Introduction

The mitochondrial F_0F_1-ATP synthase uses the transmembrane proton gradient ($\Delta\mu_{H^+}$) built up during substrate oxidation to form ATP from ADP and P_i [1]. This enzyme complex consists of two parts: a hydrophilic part, F_1, which contains the catalytic site of ATP synthesis and a membrane part, F_0, which is responsible for proton translocation through the membrane (see Ref. 2 for review). F_1 has a well-defined subunit composition, $\alpha_3\beta_3\gamma\delta\epsilon$. In addition, a protein inhibitor, IF_1, can be bound to F_1. In contrast, the nature and number of subunits constituting F_0 are not well characterized in eukaryotes. In rat liver, little is known about these F_0 subunits. In bovine heart, F_0 may contain 6 to 8 different subunits [3–6], the electrophoretic mobility of which is different from the rat liver ones: in bovine heart F_0F_1, the oligomycin-sensitivity-conferring protein (OSCP) [7] and the coupling factor F_6 [8] involved in the connection of F_1 to the membrane, the DCCD-binding protein [9] involved in proton translocation, the mtDNA encoded subunit 6 and 8 [10] and the so-called *b* (24 kDa), *d* (19 kDa) and possibly *e* subunits [5,6], have been identified. Beef-heart subunit 6, subunit *b* and the DCCD-binding protein are homologous to the *E. coli* subunits a, b and c, respectively. These three subunits are the only ones present in *E. coli* F_0 [2]. Mutations on these *E. coli* subunits can impair proton translocation. Therefore, the beef-heart subunits 6 and *b* as well as the DCCD-binding protein should be involved in proton translocation if the homologous beef heart subunits work as in *E. coli*. To our knowledge, no function is known for subunits 8, *d* and *e*. Beef-heart OSCP could be equivalent to the *b* subunit [7,11] or to the δ subunit of *E. coli* F_1 [7]. The F_0F_1-ATP synthase purified from bovine heart by different procedures has been shown to contain all or most of the proteins mentioned above, but it is not known whether these proteins are functional or structural F_0 components or if they are contaminants. The problem is particularly acute for those

Abbreviations: SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; DCCD, dicyclohexylcarbodiimide; CHAPS: 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; F_1, part of the mitochondrial adenosine triphosphatase that can be solubilized. F_0F_1 complex, mitochondrial ATPase-ATPsynthase (EC 3.6.1.3); OSCP, oligomycin-sensitivity-conferring protein.

The protein sequence data in this paper have been submitted to the NBRF-PIR Protein Sequence Database under accession number 33160. Prefix letters: γ, A (A33160, etc.); δ, B; ϵ, C; subunit *b*, D; OSCP, E; F_6, F; 9 kDa, G.

Correspondence: C. Godinot, LBTM-CNRS, Université Cl. Bernard de Lyon I, F-69622 Villeurbanne, France.

subunits which have no equivalent in *E. coli*. Therefore, it is essential to know which proteins are present in F_0F_1 preparations other than the beef heart one.

The rat liver F_0 preparation recently described by McEnery et al. [12,13] contained 7 to 8 subunits: a cluster of large polypeptides (28, 23 and 20 kDa) was assumed to correspond to bovine heart subunit *b* (24 kDa), OSCP (21 kDa) and subunit *d* (19 kDa), respectively and another cluster of small polypeptides (13.5, 11, 8–10 and 7 kDa) could be detected on gel electrophoresis, using the Laemmli procedure [14]. Nevertheless, further experimentation was necessary to demonstrate the identity of these bands and to establish which of these subunits were structural or functional components of rat liver F_0. The 8–10 kDa component could be labeled with [^{14}C]DCCD and should therefore contain the DCCD binding protein.

In another recent paper, Muraguchi et al. [15], using an electrophoretic system containing urea, identified, in rat liver, the mtDNA-encoded subunit 8 (named Chargerin II by these authors) with an apparent molecular mass lower than 6 kDa. The same group [16] purified rat liver subunit *b* by HPLC from a new (unpublished) F_0F_1 preparation and sequenced the corresponding cDNA. The amino-acid sequence deduced from this cDNA sequence gave a molecular mass of 24628 Da for the rat liver subunit *b* and was highly homologous to that of bovine subunit *b*.

In the present work, the rat liver F_0F_1-ATP synthase, purified according to McEnery et al. [12], has been compared to that of the immunoprecipitated complex by SDS-PAGE. The subunits common to the purified and immunoprecipitated complex, which were assumed to be authentic components of the complex, have then been identified either by radiolabeling or by homology of their N-terminal sequences with those known for the beef-heart complex. These identifications are presented here, to clarify our knowledge of the subunit composition of the rat liver F_0F_1-ATP synthase.

Materials and Methods

The procedure of McEnery et al. [12] was used to purify F_0F_1-ATP synthase from rat liver, using CHAPS as detergent, and that of Serrano et al. [3] was used to purify F_0F_1-ATP synthase from pig heart, using cholate as detergent. Rat liver F_1-ATPase was purified as described previously [17]. An anti-F_1 serum was obtained by injecting to rabbits with 0.5 mg of rat liver F_1 (emulsified in complete Freund adjuvant) and, 3 weeks later, 0.25 mg of F_1 (emulsified in incomplete Freund adjuvant). The sera were collected 3 days later. The booster injection and blood collection were repeated twice 1 and 2 months later without change in the serum titers. The serum was dialyzed overnight at 4°C, against 2 mM EDTA, 50 mM Tris-HCl (pH 7.5) and centrifuged at $100000 \times g$ for 30 min before use. The immunoprecipitation of F_0F_1 with the anti-F_1 serum was performed at 4°C as follows: rat liver mitochondria (2 mg protein) were lyzed for 30 min in 1 ml of 0.2% Triton X-100, 0.1 M KCl, 2 mM EDTA, 1.5 mM PMSF, 1.5 mM ATP, 50 mM Tris-HCl (pH 7.5), centrifuged for 30 min at $100000 \times g$. The lysate (0.1 ml) was preincubated for 5 h with 0.25 ml of a rabbit serum which did not precipitate the F_0F_1 complex, the final concentration of Triton X-100 and KCl being maintained at 0.2% and 0.1 M. After centrifugation at $15000 \times g$ for 15 min, the supernatant was incubated overnight with 0.3 ml of anti-F_1 serum, in the presence of 0.2% Triton X-100 and 0.1 M KCl. The immunoprecipitate collected after centrifugation at $15000 \times g$ for 30 min was washed three times with 1% Triton X-100, 0.1 M KCl, 2 mM EDTA, 50 mM Tris-HCl (pH 7.5) and once with distilled water.

SDS-PAGE was performed according to Laemmli [14] essentially as detailed in Ref. 18: the only difference was that the samples were depolymerized in the absence of urea. Two-dimensional electrophoresis was made by using non-equilibrium pH-gradient gel electrophoresis (NEpHGE) [19] in the first dimension and SDS-PAGE [14] in the second dimension. After electrophoresis, gels were stained either with Coomassie blue or silver nitrate [20] or electrotransferred to Immobilon P^{TM} for N-terminal sequence determination [21].

Results and Discussion

Compared electrophoretic profiles of purified or immunoprecipitated F_0F_1 complexes

Fig. 1A shows the electrophoretic profiles obtained for F_0F_1 preparations purified either from rat liver or from pig heart mitochondria, using either CHAPS or cholate as detergent during the step of mitochondrial membrane solubilisation. As expected, the electrophoretic profile of rat liver F_0F_1 is very similar to that described by McEnery et al. [13]. According to these authors, rat liver F_0, which is completely functional in proton translocation, only contains peptides of apparent molecular masses lower than 28 kDa. Therefore, if one excepts α, β and γ, which belong to F_1, all bands seen in Fig. 1A, lane 1, with apparent molecular masses higher than 28 kDa should be contaminants or aggregates. The bands corresponding to the large polypeptide cluster with apparent molecular masses of 28, 24 and 20 kDa do not migrate exactly as do those of pig heart F_0F_1 (lane 2). Similarly, in the small polypeptide cluster of rat liver F_0F_1, the major band migrating with an apparent molecular mass of 9 kDa did not comigrate with any pig heart F_0F_1 band. Therefore, a simple comparison of rat liver and pig heart F_0F_1 did not permit the identification of rat liver F_0 components. It should be mentioned that the electrophoretic profile of

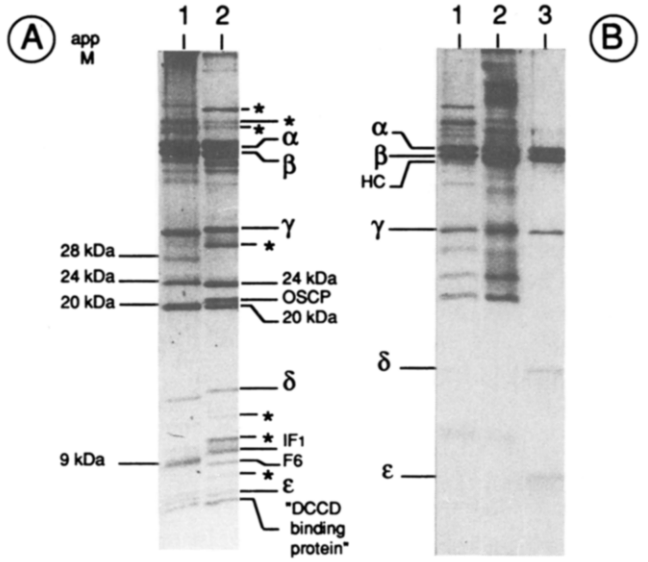

Fig. 1. (A) Compared rat liver (lane 1) and pig heart (lane 2) electrophoretic profiles of F_0F_1 purified by extraction with detergents. (B) Rat liver F_0F_1 complex purified (lane 1) and immunoprecipitated (lane 2) and rat liver purified F_1 (lane 3). Conditions as described in Methods. The gels were stained with Coomassie blue. The pig heart F_0F_1 subunit identification was performed by comparison with isolated subunits as in Ref. 18. The identification of F_6, made with isolated F_6, and of the DCCD binding protein, made by radiolabeling, are from unpublished observations (Penin, F.).

beef-heart F_0F_1 prepared as complex V [4] could not be distinguished from that of pig heart F_0F_1 (Penin, F. and Godinot, C., unpublished data).

Fig. 1B compares the electrophoretic profiles of rat liver F_0F_1 complex obtained after extraction with CHAPS or after immunoprecipitation with an antibody raised against rat liver F_1. The subunits present in both systems are likely to be authentic components of the complex. The 24, 20 and 9 kDa polypeptides have identical migrations in both preparations, while the 28 kDa polypeptide does not seem to be present in the immunoprecipitated F_0F_1 complex at the same position as in the purified one.

The differences between purified and immunoprecipitated F_0F_1 complex observed in the large polypeptide cluster area are better shown in two-dimensional electrophoresis (Fig. 2). The main 28 kDa spot seen in purified F_0F_1 (Fig. 2B) marked as II in Fig. 2D is not present in immunoprecipitated F_0F_1 (Fig. 2C). The immunoprecipitated F_0F_1 (Fig. 2C) contains several spots (spots marked IgGl, I and I′) not related to F_0F_1 that should correspond to the light immunoglobulin chains which migrate in this region of the gel. The 20 kDa band contains, both in purified (Fig. 2B) and immunoprecipitated (Fig. 2C) F_0F_1 complex, at least two spots: a large spot on the basic side which is OSCP (see below) and another well focused spot marked as 20 kDa.

N-terminal sequences of rat liver F_0F_1 subunits

The N-terminal sequences of the rat liver α and β subunits being known [22,17], only the N-terminal sequences of the polypeptides of the purified rat liver F_0F_1

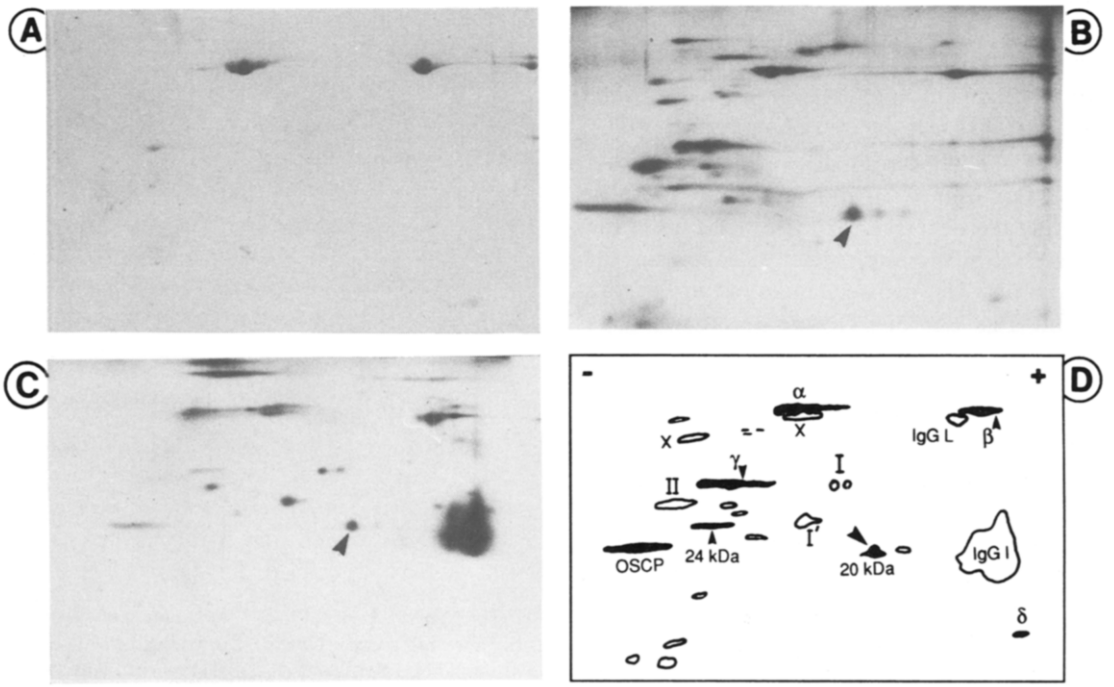

Fig. 2. Two-dimensional electrophoretic profiles of rat liver F_1 and F_0F_1. (A) Coomassie blue staining of F_1. (B) Silver staining of purified F_0F_1. (C) Silver staining of immunoprecipitated F_0F_1. (D) Synthetic interpretation of F_0F_1 spots observed in A, B and C. IgGL and IgGl: heavy and light chains of immunoglobulins. X: unidentified protein. I and I′: proteins present only in immunoprecipitated F_0F_1; II: protein present only in purified F_0F_1. In B and C, the arrow indicates the 20 kDa spot (see text).

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                10          20
"γ"r: ATLKDITRRL KSIKNIQXIT→
"γ"b: .......... ..........→

                10          20          30
"δ"r: AEAAAAPASA AGPGQMSFTF ASPTQVXFNG ANVRQVD→
"δ"b: ......Q.P. .......... ......F..S .......→

                10          20
"ε"r: VAYWRQAGLS YIRFSQIXAX AXVR→
"ε"b: .......... ...Y...C.. .VRD→

                                10
"28 kDa"r1 : (A)QTAA(A)(A)(A)PR IKTFAIYRM→ unidentified
                    10
"28 kDa"r2 : XXNKXVLVEK XGXAG→  unidentified (traces)

                    10
"24 kDa"r :   XLPPLPEYGG XVRLXLXPE→
"subunit b"b : PV.....H.. K..FG.I..→

                   10          20
"20 kDa"r : FSKLVRPPVQ VYGIEGRYAT ALYSAASKQ→
"OSCP"b :  .A........ I...Q..... .........→

                    10
"9 kDa"r1 : XKELDPVQKX FXDK→
"F6"b :     N........L .V..→

                    10
"9 kDa"r2 : VXPVQVSPLI KFGR→ unidentified
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Fig. 3. N-terminal sequences of rat liver F_0F_1 subunits. The sequences of the rat liver (r) F_0 subunits are compared to those known for beef heart (b). For the 28 and the 9 kDa bands, two amino acids were present at each sequencing cycle, in very different amounts, thus permitting the distinction between the two sequences r_1 and r_2. Points indicate identity between the beef heart and rat liver sequences. When amino acids are indicated in parentheses, there is some uncertainty. X = unidentified amino acid.

complex, with M_r lower than 30000, have been determined (Fig. 3). The N-terminal sequences obtained for the small F_1 subunits γ, δ and ϵ are very close to those obtained for the beef-heart enzyme, in spite of the differences observed in the electrophoretic migration of the γ and δ subunits. The two sequences obtained from the 28 kDa band (Fig. 1A, lane 1) were not homologous to any beef-heart F_0 protein (or to any protein present in the NBRF or Swissprot bank). The 24 kDa band has the same sequence as that published by Tsurumi et al. [16] for isolated rat liver subunit *b* and is homologous to the beef-heart subunit *b* [5]. The only sequence obtained from the 20 kDa band is very close to the beef-heart OSCP sequence. This is to our knowledge the first report of the N-terminal sequence of OSCP, γ, δ and ϵ subunits. An N-terminal sequence identical to that of OSCP was found when sequencing the 20 kDa spot present on the basic side in two-dimensional electrophoresis. This indicates that this spot corresponded to OSCP. No sequence could be obtained for the other 20 kDa protein visible in two-dimensional electrophoretic gels of immunoprecipated or purified F_0F_1 complex, which suggests that this protein has a blocked N-terminal sequence. The subunit *d* present in beef-heart F_0F_1 complex also had a blocked N-terminal sequence and has an M_r of 18603. It could be a good candidate to be equivalent to the unidentified 20 kDa protein. However, this remains to be confirmed. The 9 kDa band gave two sequences, one homologous to beef-heart F_6, which demonstrates the presence of F_6 in the rat liver enzyme. This sequence is also identical to that very recently obtained by Higuti et al. [23]. The other sequence found at the level of the 9 kDa band does not to our knowledge correspond to any sequence reported.

F_0 subunits of mitochondrial origin

The mitochondrial DNA codes for the subunits 6 and 8 of F_0. Their M_r calculated from the rat liver mtDNA sequence [10] are 25033 and 7636, respectively. Since they are hydrophobic, they are difficult to stain and may have anomalous migration in gel electrophoresis. In addition, many proteins of mitochondrial origin have a blocked N-terminal amino acid. For these reasons, subunits 6 and 8 might have escaped our above analyses. Their identification has been performed first by labeling the mitochondrial proteins in vitro with [^{35}S]methionine in the presence of cycloheximide to prevent the labeling of proteins of nuclear origin, then, by immunoprecipitating the F_0F_1 complex with the anti-

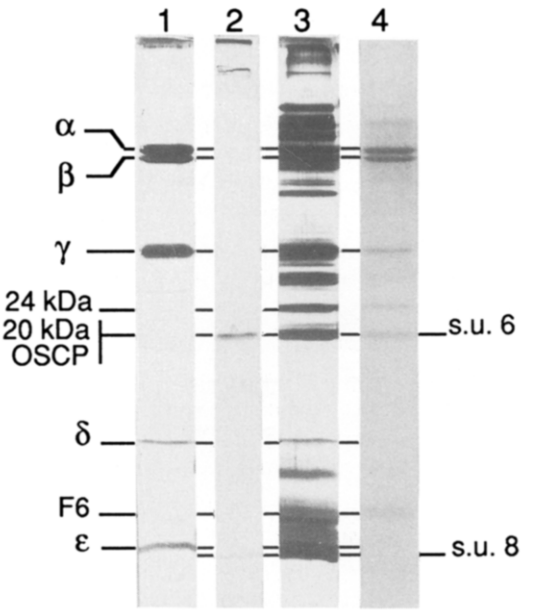

Fig. 4. Separation of rat liver F_0F_1 subunits of mitochondrial origin in the Laemmli system. Lane 1, silver-stained F_1. Lane 2, autoradiogram of immunoprecipitated F_0F_1 labeled in vitro with [^{35}S]Met, as in Ref. 24. Only the mitochondrially synthesized subunits are labeled, since the labeling was performed in the presence of cycloheximide which inhibits protein synthesis of nuclear origin. Lane 3, silver stained purified F_0F_1. Lane 4, Coomassie blue stained purified F_0F_1. s.u. 6 and 8: subunits 6 and 8.

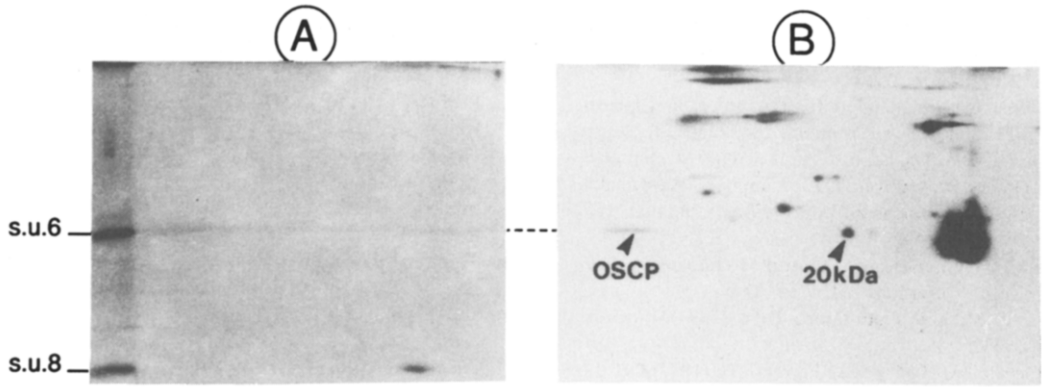

Fig. 5. Two-dimensional electrophoresis of radiolabeled subunits 6 and 8 in rat liver F_0F_1. The acid side is on the right and the basic side on the left, as in Fig. 3. (A) Autoradiogram of immunoprecipitated F_0F_1. A sample of immunoprecipitated F_0F_1 (visible on the left) was run in the second dimension as a marker. (B) Silver staining of immunoprecipitated F_0F_1.

F_1 serum and finally by autoradiography of the gel electrophoresis prepared with this immunoprecipitate. Fig. 4 shows that the presence of these two subunits can be demonstrated at the level of the 20 kDa band and in front of the ϵ subunit of F_1. Fig. 5 shows that subunit 6 is different from the 20 kDa protein discussed above. Indeed, while subunit 8 is well focused at the acid side of the gel, the radioactivity incorporated into subunit 6 is scattered all along the gel width (Fig. 5A). OSCP was focused on the basic side and the 20 kDa protein was focused about in the middle of the gel. Therefore, there are three subunits at the level of the 20 kDa band.

Conclusion

In this study, we have shown that the rat liver F_0F_1 complex prepared either by purification, using CHAPS as detergent [12] or by immunoprecipitation with an anti-F_1 serum contains, in addition to the F_1 subunits: a 24 kDa polypeptide homologous to subunit *b* in beef heart; a 20 kDa band which contained three different polypeptides corresponding to OSCP, the subunit 6 of mitochondrial origin and another 20 kDa protein that might be equivalent to the subunit *d* described for beef-heart; a 9 kDa band containing a protein with an N-terminal sequence similar to the beef-heart F_6; the subunit 8 of mitochondrial origin migrating in front of the ϵ subunit of F_1.

The 28 kDa band found in the purified F_0F_1 complex but not in the immunoprecipitated complex, containing proteins of sequence unrelated to any known protein might be a contaminant even though it was found in large amount in F_0 preparations [13]. These sequences did not correspond to the sequences known for the ATP/ADP, P_i or substrate translocators known to migrate as 25 to 30 kDa proteins. At least two additional proteins identified in previous studies should be added to this list: the DCCD binding protein which migrates as a 8–10 kDa protein in the same electrophoretic system [13] and the protein inhibitor IF_1 which migrates as a 12.5 kDa protein [25]. The results indicate that the total rat liver F_0F_1 complex with its regulatory inhibitor contains at least 13 polypeptides, seven of which can be attributed to F_0.

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