Biochimica et Biophysica Acta, 724 (1983) 230–240
Elsevier

BBA 41346

IMMUNOCHEMICAL ANALYSIS OF *MICROCOCCUS LYSODEIKTICUS (LUTEUS)* F_1-ATPase AND ITS SUBUNITS

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(Received March 7th, 1983)

Key words: F_1-ATPase; Immunoelectrophoresis; (Micrococcus lysodeikticus)

The F_1-ATPase from *Micrococcus lysodeikticus* has been purified to 95% protein homogeneity in this laboratory and as all other bacterial F_1s, possesses five distinct subunits with molecular weights ranging from 60000 to 10000 (Huberman, M. and Salton, M.R.J. (1979) Biochim. Biophys. Acta 547, 230–240). In this communication, we demonstrate the immunochemical reactivities of antibodies to native and SDS-dissociated subunits with the native and dissociated F_1-ATPase and show that: (1) the antibodies generated to the native or SDS-dissociated subunits react with the native molecule; (2) all of the subunits comprising the F_1 are antigenically unique as determined by crossed immunoelectrophoresis and the Ouchterlony double-diffusion techniques; (3) antibodies to the SDS-denatured individual δ- and ϵ-subunits can be used to destabilize the interaction of these specific subunits with the rest of the native F_1; and (4) all subunit antibodies as well as anti-native F_1 were found to inhibit ATPase activity to varying degrees, the strongest inhibition being seen with antibodies to the total F_1 and anti-α- and anti-β-subunit antibodies. The interaction of specific subunit antibodies may provide a new and novel way to study further and characterize the catalytic portions of F_1-ATPases and in general may offer an additional method for the examination of multimeric proteins.

Introduction

The F_1-ATPases from several sources have been purified to protein homogeneity and in general possess five distinct subunits designated α-, β-, γ-, δ- and ϵ- in order of their decreasing molecular weights [1,2]. The β-subunit of these enzymes contains the site(s) for catalytic activity [1] and reconstitution experiments with α-, β- and γ-subunits from the same [1] or hybrid ATPases [3] have shown that these subunits are necessary for ATPase activity. The δ- and ϵ-subunits serve as structural proteins for attachment of the F_1s to the F_0 portion in the membrane [2]. A role for the ϵ-subunit as a natural inhibitor has also been suggested [2].

We have recently reported the purification of the latent F_1-ATPase from *Micrococcus lysodeikticus* and showed that it also contains five different subunits [4]. Earlier work from this laboratory has shown that the ATPase from this organism accounts for about 10% of the total protein of isolated cell membranes [5] and represents a major immunoprecipitate when membranes are solubilized with the nonionic detergent Triton X-100 and analysed by the two-dimensional, high-resolution procedure of crossed immunoelectrophoresis [6].

In the studies reported in this communication, we have generated antibodies to several forms of the native and denatured F_1-ATPase as well as to the individual SDS-dissociated subunits. Using the techniques of crossed immunoelectrophoresis and in vitro inhibition assays, we have investigated the structural and functional properties of the *M. lysodeikticus* F_1-ATPase and its subunits.

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Materials and Methods

M. lysodeikticus (M. luteus, NCTC 2665) was grown in peptone/water/yeast extract medium (medium A) the cells harvested, washed and lysed [4,7] and the resulting membranes were then shocked as previously described to release the F_1-ATPase [4]. The purification scheme for the F_1-ATPase of *M. lysodeikticus* was followed exactly as described by Huberman and Salton [4].

Preparation of antiserum. Antisera to *M. lysodeikticus* membranes, Triton X-100-solubilized extracts of membranes and to purified F_1-ATPase were obtained by subcutaneously injecting into New Zealand White rabbits 0.5 ml of a solution of 1–2 mg protein/ml with an equal volume of Freund's incomplete adjuvant. This procedure was repeated once a week for 5 weeks. 10 days after the last injection, the rabbits were bled as previously described [8].

Antiserum to the denatured F_1-ATPase was made in two separate ways. Owen and Salton [6] generated antiserum to an ATPase preparation that was fully dissociated by treatment with urea, dithiothreitol and iodoacetamide. Antiserum was also made to an SDS-denatured F_1-ATPase by adding SDS to purified F_1-ATPase (0.2% final concentration) and then dialysing overnight into 50 mM Tris-HCl buffer, pH 7.5, in the cold. Injection and bleeding procedures were performed as described above.

Antibodies to the α-, β-, γ-, δ- and ϵ-subunits were obtained in the following way. Approx. 400 μg of purified F_1-ATPase were run on a 10–13% gradient gel using the Laemmli SDS-polyacrylamide gel electrophoresis system [4,10]. When the tracking dye reached the bottom of the gel, the whole gel was placed in distilled water for 10 min, and then stained with 0.25% aqueous Coomassie brilliant blue R-250 stain for 20–30 min. The gel was then destained with several changes of distilled water. Each of the zones of the gel containing α- and β-, γ-, δ- and ϵ-subunits were separately excised and then placed into dialysis sacs with 1–2 ml of 50 mM Tris-HCl buffer, pH 7.5. Dialysis tubing with a 10000 M_r cutoff was used for all of the subunits except the ϵ-subunit. For this we used Spectrapor (Spectrum Medical Ind., Inc., Los Angeles, CA) tubing with a 6000-8000 M_r cutoff. The sacs were then dialysed against 61 of 50 mM Tris-HCl buffer, pH 7.5, overnight in the cold. After dialysis, the samples were homogenized with a mortar and pestle and 0.5-ml volumes dispensed into tubes for storage and use. When required, samples were mixed with equal volumes of Freund's incomplete adjuvant and injected subcutaneously into New Zealand White rabbits using the same immunization schedule as described above. The γ-subunit preparation was also injected into four Hartley guinea pigs. Since the α- and β-subunits could not be separated far enough apart in the Laemmli SDS-polyacrylamide gel electrophoresis system for clean excision, antibodies were first generated to the α- plus β-subunit portion of the gels. When either the α- or the β-subunit was excised individually from the SDS-polyacrylamide gel electrophoresis gel there was always a small portion of the other subunit present which could be detected when subjected to rocket or crossed immunoelectrophoresis. When excised α- or β-subunit was electrophoresed into anti-denatured α- plus β-subunit antibodies by the rocket immunoelectrophoresis method, the individual peaks were cut out of the immunoplates and then used as immunogens and under these conditions pure anti-α- or anti-β-subunit antibodies were thus obtained. Specific antisera were pooled after a minimum of six bleeds and processed according to the procedure of Harboe and Ingild [11] but were not passed through a DE52 column. Pooled, processed sera were concentrated between 5- and 10-times with respect to starting volumes.

SDS-polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis was performed using the Laemmli buffer system and stained with Coomassie brilliant blue R-250 [10]. The solubilization of immunoprecipitates for subsequent SDS-polyacrylamide gel electrophoresis analysis was performed with a slight modification from the method of Norrild et al. [12]. Since the ATPase immunoprecipitates were quite visible on the immunoplates and since our antibodies and antigens were specific and 95% homogeneous, respectively, immunoprecipitates were excised from a pressed immunoplate and solubilized with the Laemmli solubilization buffer. After boiling for 2 min, samples were kept in hot water and were applied to an SDS gel with a Hamilton syringe also kept in hot water.

Immunoelectrophoretic techniques. Crossed immunoelectrophoresis [8] and zymogram staining [6] were performed as previously described from this laboratory [11]. Briefly, antigen was electrophoresed in the first dimension for 60 min at 15 mA (10°C) and into the second dimension for 16 h at 55 V (10°C). At the end of an electrophoretic run immunoplates were soaked in 0.1 M NaCl and pressed several times to remove unreacted proteins [8]. The gels were either dried down and stained with 0.25% Coomassie brilliant blue or subjected to the ATPase zymogram stain. Where the intermediate gel variation of the crossed immunoelectrophoresis method [13] was used, appropriate volumes of either 50 mM Tris-HCl buffer, pH 7.5, or preimmune immunoglobulins were added to intermediate gel blanks as controls. For rocket immunoelectrophoresis, antigen was electrophoresed directly into gels containing antibody at 2.5 V/cm for 16 h [14]. Tandem immunoelectrophoresis was performed as described by Krøll [15]. For the Ouchterlony double-diffusion experiments, wells were punched into agarose slides and antibody and antigen applied. Gels were then placed in a moist chamber for 48 hr at room temperature and processed in a similar manner to the other immunoplates.

Assay of ATPase activity. To determine ATPase activity, the liberation of P_i was measured colorimetrically by the method of Fiske and Subbarow [16] as previously described [4].

Autoradiography. The immunoplates were soaked and pressed as described, dried and then subjected to autoradiography for 23 days at −70°C using Kodak XR-5, X-O Mat film.

Protein determination. Protein was determined by the method of Lowry et al. [17] using bovine serum albumin as a standard.

Antibody inhibition assays of ATPase activity. To study the inhibitory effects of the various antisera 2–6 μg of purified F_1-ATPase were incubated with 250 μl of specific antiserum. The antisera which were normally kept in 0.1 M NaCl and 15 mM NaN_3 were dialyzed overnight against 6 l of 50 mM Tris-HCl buffer, pH 7.5, in the cold prior to incubation with the ATPase for 1 h at room temperature (25°C). The reaction mixtures were then placed in an ice bath to which 4 μmol Na_2ATP, 4 μmol $CaCl_2$, 37.5 μmol Tris-HCl buffer, pH 7.5, and 150 μg trypsin were added to a final volume of 0.5 ml. Since these assays were performed both in the presence and absence of trypsin, an appropriate volume of 100 mM Tris-HCl buffer was added to those tubes assayed in the absence of trypsin. After 10 min incubation at 37°C, an equal volume of 10% (w/v) trichloroacetic acid was added to each tube and the various incubation mixtures were allowed to stand in the cold for 1 h prior to centrifugation at 12000 rpm for 15 min. ATPase activity was determined by assaying the supernatant fluids for P_i as previously described [4]. Specific antiserum controls (i.e., substrate, Ca^{2+}, no enzyme) were performed for each antibody used.

Radioactive labeling of cells. Labeled cells of *M. lysodeikticus* were prepared by growing cells in 1 : 5 diluted medium A supplemented with either 125 μCi of ^{14}C-labeled algal protein hydrolysate (Amersham, Arlington Heights, IL) or 200 μCi of [^{35}S]methionine (New England Nuclear, Boston, MA). Cells were harvested and purified ATPase was prepared as previously described [4].

Chemicals. Agarose, type HGT, was obtained from Marine Colloids, Rockland, ME. All other chemicals of highest grades were obtained from standard commercial sources and were used without further purification.

Results

As with all other bacterial F_1-ATPases [1] and with F_1s in general, the SDS profile of purified F_1-ATPase from *M. lysodeikticus* contained five distinct subunits designated α-, β-, γ-, δ- and ε- in order of decreasing molecular weights (Fig. 1). Even when run on a 10–13% gradient Laemmli gel system, the α- and β-subunits ran quite close to-

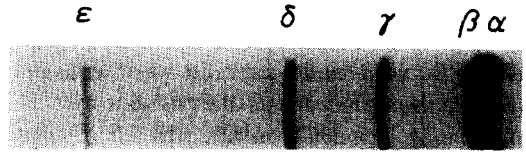

Fig. 1. SDS-polyacrylamide gel electrophoresis profile of 40 μg of purified F_1-ATPase from *M. lysodeikticus*, stained with Coomassie brilliant blue. A 10–13% gradient gel was used and run according to the Laemmli system.

gether and it was for this reason that antibodies were initially generated to the α- plus β-subunit portion of the gel and then to the individual α- and β-subunits using rocket immunoelectrophoresis as described in Materials and Methods.

Immunoprecipitates formed with subunits and homologous antisera

Proteins eluted from SDS gels have been shown to be both antigenic and immunogenic [18,19]. The series of slides shown in Fig. 2 demonstrate the immunoprecipitate(s) formed when excised and eluted α- plus β-, γ-, δ- and ϵ-subunits were subjected to crossed immunoelectrophoresis with their homologous antiserum (Fig. 2a–d, respectively). The same pattern(s) were seen when the purified F_1-ATPase from *M. lysodeikticus* was denatured with SDS at a final concentration of 0.1% (SDS/protein ratio of 1:3) and subjected to crossed immunoelectrophoretic analysis (data not shown). It is interesting to note that the electrophoretic mobilities of the individual subunits were substantially less than that of the purified F_1-ATPase (cf. Fig. 2a–d with Fig. 2e).

Various subunit antibodies can be added to the upper portion of the reference gel to obtain a composite crossed immunoelectrophoretic pattern for the SDS-dissociated F_1-ATPase. It was technically difficult to obtain one immunoplate containing all five immunoprecipitate subunits comprising the F_1-ATPase, since different antibody/antigen ratios are necessary to form them. Therefore, a schematic composite crossed immunoelectrophoretic reference pattern of subunits is shown in Fig. 3. Since we have started with a 95% homogeneous protein and then used the SDS-dissociated subunits as immunogens, we believe that we have obtained monospecific antisera to each of the subunits.

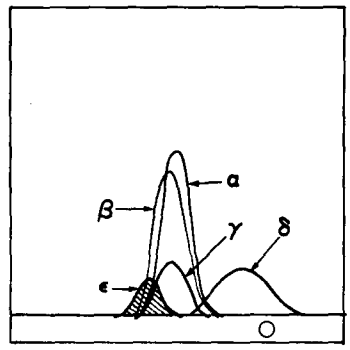

Fig. 3. This composite figure diagrammatically demonstrates the five distinct immunoprecipitates seen when the SDS-dissociated F_1-ATPase subunits are electrophoresed into their homologous anti-subunit antisera.

Reactivities of specific subunit antisera with the native F_1-ATPase

The use of the intermediate gel technique has been quite valuable in establishing immunological relationships (e.g., identity, nonidentity, cross-reactivity) [20] and the results presented in the panel of slides shown in Fig. 4 confirm the antigenic uniqueness of each of the F_1 subunits. In these slides, native, purified F_1-ATPase was electro-

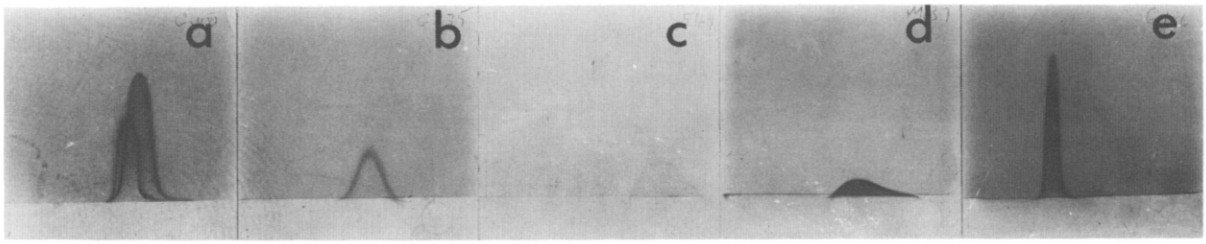

Fig. 2. Crossed immunoelectrophoretic analysis of excised and eluted F_1-ATPase subunits from an SDS-polyacrylamide gel run against their homologous antiserum. Antigens were dialyzed into 50 mM Tris-HCl buffer, pH 7.5, in the cold overnight, and were then electrophoresed for 60 min at 15 mA in the first dimension and for 16 h and 55 V in the second dimension. (a) Approx. 1.6 μg α- plus β-subunit antigen and 400 μl of 5-fold concentrated anti-α- plus β-subunit antibodies. (b) Approx. 180 ng γ-subunit antigen and 300 μl of 5-fold concentrated anti-γ-subunit antibodies. (c) Approx. 130 ng δ-subunit antigen and 200 μl of 5-fold concentrated anti-δ-subunit antibodies. (d) Approx. 120 ng ϵ-subunit antigen and 400 μl of 5-fold concentrated anti-ϵ-subunit antibodies. (e) 2 μg purified native F_1-ATPase and 200 μl anti-α- plus β-subunit antibodies. Slides were stained with Coomassie brilliant blue stain.

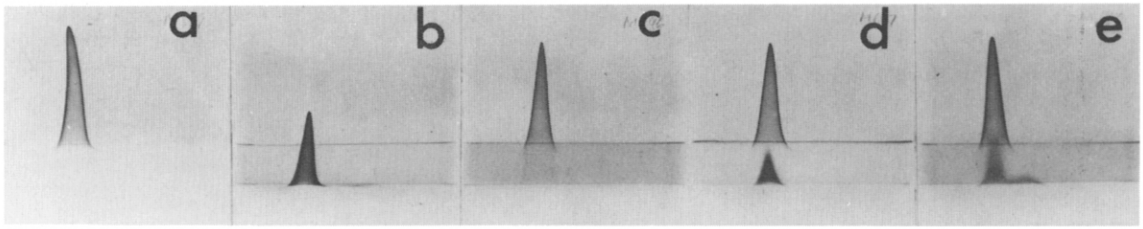

Fig. 4. F_1-ATPase subunit analysis using the intermediate gel technique. 2.25 μg of purified F_1-ATPase were electrophoresed for 60 min in the first dimension at 15 mA and then for 15 h at 55 V into the second dimension in which the antibody reference gels contained 100 μl of anti-purified F_1-ATPase antibodies. The intermediate gels contained (a) no antibodies, (b) 200 μl anti-α- plus β-subunit antibodies, (c) 200 μl anti-γ-subunit antibodies, (d) 200 μl anti-δ-subunit antibodies, (e) 200 μl anti-ϵ-subunit antibodies. Immunoplates were stained with Coomassie brilliant blue stain.

phoresed into an anti-membrane antibody reference system through intermediate gels which contained the various anti-subunit antisera (Fig. 4a represents the control). The interposition of anti-α- plus β-subunit antibodies into the intermediate area of the gel largely depressed the immunoprecipitate of native F_1-ATPase into this zone (Fig. 4b) and could depress the entire immunoprecipitate if larger amounts of antibodies had been used. Precipitation of the F_1 by α- and β-subunit antibodies is not surprising, since the α- and β-subunits together constitute over 80% of the total F_1-ATPase protein. The interposition of either anti-α- or anti-β-subunit also showed the same phenomena (data not shown). A weaker precipitation reaction was observed when 200 μl of anti-γ-subunit antibodies were incorporated into the intermediate gel area but larger amounts of antiserum showed a stronger reaction (Fig. 4c). Surprisingly, the interposition of anti-δ- or anti-ϵ-subunit antibodies within the intermediate gel showed strong, distinct immunoprecipitates both

TABLE I

ANTIGENICITY AND IMMUNOGENICITY OF *M. LYSODEIKTICUS* F_1-ATPase AND ITS SUBUNITS AS DETERMINED BY CROSSED IMMUNOELECTROPHORETIC AND THE OUCHTERLONY DOUBLE DIFFUSION TECHNIQUES

The scoring system was based on several individual experiments and was judged by the staining intensity, size and number of immunoprecipitates observed. n.d., not done; ± = slight reaction detected.

Antibody	SDS-denatured F_1-ATPase	Excised α-subunit	Excised β-subunit	Excised α/β-subunit	Excised γ-subunit	Excised δ-subunit	Excised ϵ-subunit	Purified F_1-ATPase	Triton X-100-solubilized membranes
Anti-Triton X-100-solubilized *M. lysodeikticus* membranes	+ +	+	+	+	±	+	±	+ + +	+ + +
Anti-*M. lysodeikticus* membranes	+ +	+ +	+ +	+ +	±	+ +	±	+ + +	+ + +
Anti-purified F_1-ATPase	+	+	+	+	±	+	±	+ + +	+ + +
Anti-SDS-denatured F_1-ATPase	+ + +	+ + +	+ + +	+ + +	±	+ + +	±	+ +	+ +
Anti-α/β-subunit	+ + +	+ + +	+ + +	+ + +	−	−	−	+ +	+ +
Anti-α-subunit	+ + +	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	+ +	n.d.
Anti-β-subunit	+ + +	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	+ +	n.d.
Anti-γ-subunit	+ + +	−	−	−	+ + +	−	−	+	+
Anti-δ-subunit	+ + +	−	−	−	−	+ + +	−	+ + +	+ + +
Anti-ϵ-subunit	+ + +	−	−	−	−	−	+ + +	+ + +	+ +

within the intermediate gel zone and the reference area of the immunoplates (Fig. 4d and e, respectively). Note that the peak heights of the reference immunoprecipitates in Fig. 4d and e were not changed significantly from that of the control F_1 immunoprecipitate shown in Fig. 4a.

Antigenicity of the F_1-ATPase and its subunits

When SDS-denatured F_1-ATPase was tested against an anti-membrane reference system or against anti-SDS-dissociated F_1-ATPase reference antibodies, only the α-, β- and δ-subunit immunoprecipitates were present and were also formed when any of these three antisera were incorporated into the intermediate gel zone of the immunoplate. The γ- and ϵ-subunit immunoprecipitates formed only in the intermediate gels containing their respective antibodies, indicating the lack of detectable antibodies to these subunits in the reference anti-membrane antiserum [20]. The results of the reactions of the native, SDS-denatured and individual dissociated subunits of the F_1 against the various antisera are summarized in Table I and were determined by crossed, rocket, co- and tandem immunoelectrophoresis as well as by the Ouchterlony double-diffusion technique and show, moreover, that each of the subunits is antigenically unique.

Separation of the ϵ-subunits during electrophoresis

During a first-dimensional run in crossed immunoelectrophoresis, proteins are separated according to their electrophoretic mobilities. Depending upon the association of the subunits in a multimeric protein such as the F_1-ATPases one might also be able to detect conformational changes. A recent investigation by Dreyfus et al. [21] has shown that an electrochemical gradient can induce a displacement of the inhibitor protein of the mitochondrial F_1-ATPase. The results in the panel of slides illustrated in Fig. 5 show that the *M. lysodeikticus* F_1-ATPase may be undergoing a change such that the ϵ-subunits are released from the complex during the course of electrophoresis for 15–60 min in the first dimension. Moreover, the slides shown in Fig. 6 suggest that the ϵ-subunit can be completely detached from the rest of the F_1-ATPase molecule upon electrophoresis under standard conditions used throughout the studies at pH 8.6 in the presence of Trition X-100 (1%).

Catalytic activity of the F_1-ATPase immunoprecipitates

Another great advantage of the crossed immunoelectrophoretic method of analysis of membrane antigens is the fact that many enzymes complexed with their antibodies still retain intrinsic biological activity and can be detected by specific enzyme staining [9]. When purified F_1-ATPase was electrophoresed into anti-membrane, anti-purified F_1-ATPase, anti-Triton X-100-solubilized membranes, anti-SDS-dissociated ATPase, anti-α- plus β-, anti-α- or anti-β-subunit antibodies, residual enzyme activity could be detected with the zymogram stain for ATPase, although as one might expect, qualitative differences in the staining intensity were observed. The slides presented in Fig. 7a show the Coomassie brilliant blue stain of a slide in which purified F_1-ATPase was

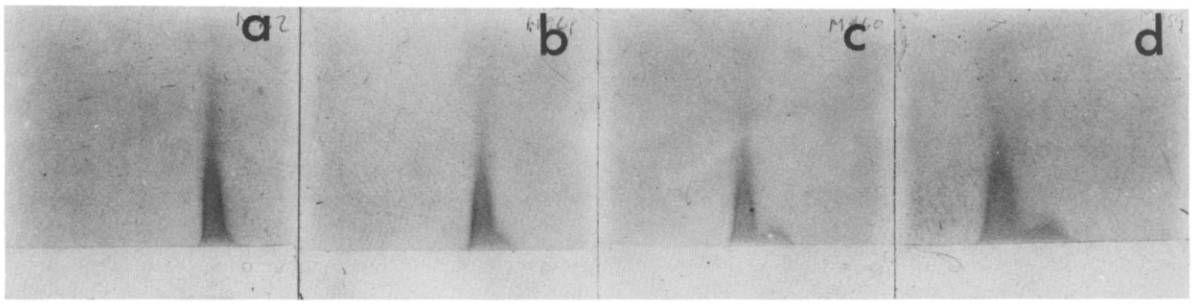

Fig. 5. 1.5 μg purified latent F_1-ATPase electrophoresed for (a) 15 min, (b) 30 min, (c) 45 min, and (d) 60 min at 15 mA. The F_1-ATPase was then electrophoresed into 400 μl of anti-ϵ-subunit antibodies at 55 V for 15 h and stained with Coomassie brilliant blue. Notice the shoulder that has formed as a result of increased electrophoresis time.

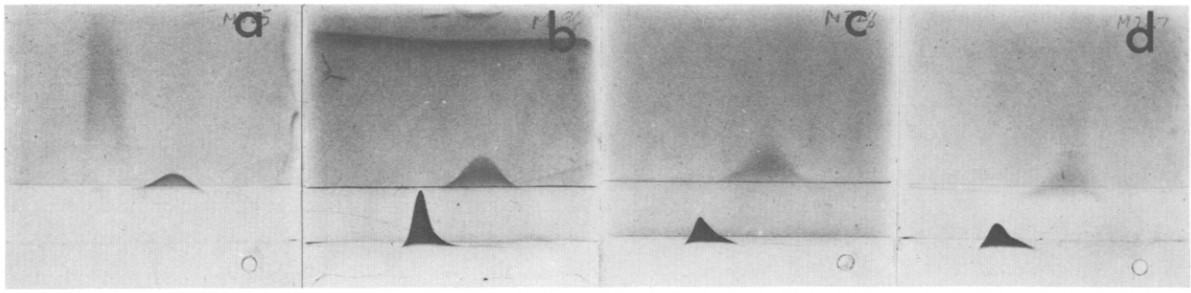

Fig. 6. 1.5 μg of purified F_1-ATPase were electrophoresed for 60 min at 15 mA in the first dimension and then for 15 h at 55 V into an anti-reference gel area containing 400 μl of anti-ϵ antibodies. Intermediate gels contained (a) no antibodies, (b) 100 μl anti-membrane antibodies, (c) 100 μl anti-purified F_1-ATPase, (d) 100 μl anti-Triton X-100-solubilized *M. lysodeikticus* membrane antibodies.

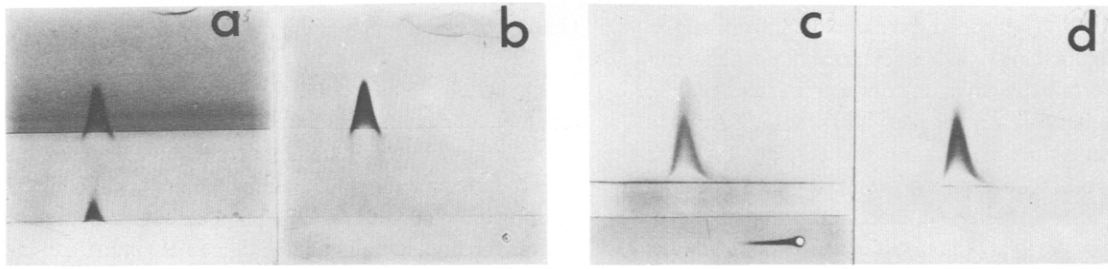

Fig. 7. (a and b) 2.0 μg of purified F_1-ATPase were electrophoresed for 60 min at 15 mA in the first dimension. I hen electrophoresed through an intermediate gel containing 200 μl anti-δ-subunit antibodies and a reference gel containing 40(i-α- plus β-subunit antibodies. Slide a was stained with Coomassie brilliant blue stain and slide b was subjected to the zymogram stain for ATPase activity as previously described [6]. (c and d) 1.0 μg of purified F_1-ATPase was electrophoresed for 100 min at 15 mA in the first dimension directly into 200 μl of anti-δ-subunit antibodies. It was then electrophoresed into 200 μl of anti-α- plus β-subunit antibodies in the second dimension. Slide c was stained with Coomassie brilliant blue and slide d was subjected to a zymogram stain for ATPase activity.

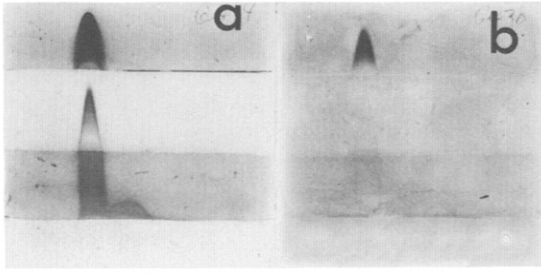

Fig. 8. 1.5 μg of purified F_1-ATPase were electrophoresed for 60 min at 15 mA in the first dimension. It was then electrophoresed through 300 μl of anti-δ-subunit, 200 μl of anti-δ-subunit and 200 μl of anti-α- plus β-subunit antibodies at 55 V for 15 h in the second dimension. Slide a was stained with Coomassie brilliant blue and slide b was subjected to a zymogram stain for ATPase activity.

first electrophoresed through anti-δ-subunit antibodies and then into anti-α- plus β-subunit antibodies. When an identical immunoplate was subjected to a zymogram stain for ATPase activity only the reference portion of the gel showed any ATPase activity (Fig. 7b). By a modification of this technique (Fig. 7c and d) it was established that when F_1-ATPase was electrophoresed in the first dimension through the agarose gel containing anti-δ-subunit antibodies and then electrophoresed in the second dimension into anti-α- plus β-subunit antibodies only the immunoprecipitate in the reference gel demonstrated ATPase activity. Again, upon electrophoresis of the F_1-ATPase sequentially through anti-ϵ-, anti-δ- and then into anti-α- plus β-subunit antibodies only the immunoprecipitate formed with the latter antibodies showed enzyme activity (Fig. 8a and b).

The immunochemical analysis of the F_1-ATPase presented in Figs. 7 and 8 raised the possibility that reaction with anti-δ- and anti-ε-subunit antibodies detaches the respective subunits from the F_1-ATPase. Accordingly, radioactively labeled F_1-ATPase was prepared in order to obtain further qualitative and quantitative evidence that the δ- and ε-subunits were detached, leaving an α- ,β-, γ-subunit complex of the F_1.

SDS-polyacrylamide gel electrophoresis and autoradiography of the F_1-ATPase after electrophoresis through intermediate gel zones

Norrild et al. [12] have shown that immunoprecipitates could be subjected to polypeptide analysis by excision and examination by SDS-polyacrylamide gel electrophoresis. This procedure therefore offered the opportunity of further analysis of the interactions of the F_1-ATPase with specific subunit antibodies. When this was performed on immunoprecipitates produced from purified ^{14}C-labeled F_1-ATPase and anti-membrane antiserum one could clearly visualize the α-, β- , γ- and δ-subunits. If the SDS gel was then sliced into 1-mm strips and counted for the production of a radioactive profile, in a typical experiment one could obtain 3100 cpm in the α- plus β-subunit region, 500 cpm in the γ-subunit region and 160 cpm in the δ-subunit region of the gel. When the same amount of protein was electrophoresed through anti-ε-, anti-δ-subunit and anti-membrane gel zones one could recover 2700 cpm in the α- plus β-subunit portions of the gel and 450 cpm in the γ-subunit portion of the gel. 90% of the expected radioactivity could be recovered from the δ-subunit region of the gel with approx. 5% of the counts recoverable in the α- plus β-subunit area. Only a small amount of radioactivity could be obtained from the ε-subunit portion of the gel when the anti-ε-subunit immunoprecipitate was analysed by SDS-polyacrylamide gel electrophoresis. These experiments were also performed in the presence of 1 mM PMSF to control for proteolysis.

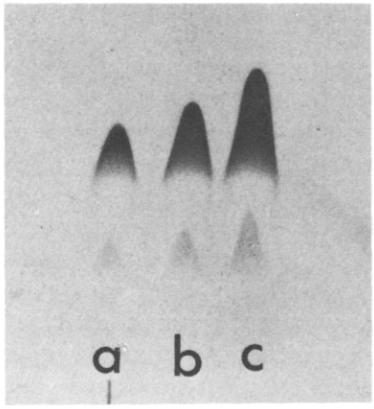

Fig. 9. 100, 200 and 400 cpm of ^{35}S-labeled F_1-ATPase corresponding to 1, 2 and 4 μg protein, respectively, were rocketed into 400 μl of anti-ε-subunit, 300 μl of anti-δ-subunit and then 400 μl of anti-α- plus β-subunit antibodies. The immunoplate was dried down and subjected to autoradiography for 23 days at −70°C using Kodak XR-5, X-O Mat film.

Fig. 9 shows the results of an autoradiogram where ^{35}S-labeled F_1-ATPase was examined by rocket electrophoresis through anti-ε-,anti-δ- and then into anti-α- plus β-subunit antibodies. Similar experiments were performed with ^{14}C-labeled F_1-ATPase. These results demonstrate a quantitative difference of label in the three immunoprecipitates which might reflect the quantitative differences of label within each SDS-denatured subunit band. The amount of protein rocketed in the autoradiogram shown in Fig. 9 was (a) 1 μg, (b) 2 μg and (c) 4 μg corresponding to 100, 200 and 400 cpm, respectively. When these immunoprecipitates were scanned for the production of densitometry profiles, the areas under the anti-δ-subunit peaks represented between 5 and 6% of the total area. Assuming a 3 : 3 : 1 : 1 : 1 stoichiometry for the bacterial F_1s [2,32], the δ-subunit represents approx. 6% of the total F_1 molecular weight. These data therefore also suggest a possible detachment of the δ-subunit from the F_1.

Antibody inhibition assays of ATPase activity

The availability of subunit-specific antisera provided an opportunity to test the effects of the individual subunit antibodies on the ATPase activity of the F_1. In contrast to the conditions of the crossed immunoelectrophoresis experiments and zymogram staining, the determination of the inhibitory activities of the antisera was performed in bulk solution. The amount of specific antibody used for our in vitro inhibition assays was equivalent to the volume necessary to form an im-

TABLE II

ANTIBODY INHIBITION STUDIES

Inhibition assays were performed as explained in Material and Methods and represent an average % inhibition by each antibody used against 2–6 μg of purified F_1-ATPase.

Antibody	% inhibition	
	+ trypsin	− trypsin
Anti-α/β-subunit	93	93
Anti-α-subunit	62	62
Anti-β-subunit	64	64
Anti-α- plus anti-β-subunit	85	75
Anti-γ-subunit	37	38
Anti-δ-subunit	41	47
Anti-ε-subunit	40	53
Anti-*M. lysodeikticus* membrane	95	91
Anti-purified F_1-ATPase	91	95
Anti-Triton X-100-solubilized membrane	96	96
Anti-SDS-denatured F_1-ATPase	97	97

munoprecipitate during immunoelectrophoresis (i.e., antibody excess). Table II shows the inhibitory effects of the various antisera generated to the whole F_1-ATPase or its subunits on the catalytic activity of the enzyme. All of the antisera inhibited ATP hydrolysis, but antibodies generated to the whole enzyme (native or denatured F_1) or to the major (α- and/or β-) subunits inhibited catalytic activity to the greatest extent.

Discussion

Many immunological techniques have been used to study the structure and function of a wide variety of bacterial F_1-ATPases and their subunits [1,2]. There appear to be very few instances to our knowledge where antibodies have been generated to all five F_1 subunits. In the recent studies of Smith and Sternweis [23] antibodies were prepared to the α- , β- , γ- , δ- and ε-subunits of *Escherichia coli* F_1-ATPase but the α antiserum reacted only with the denatured α-chain. The majority of the studies so far reported generally relate to antibodies to the α- and β-subunits and have not addressed the reactivity of specific antisera generated to SDS-dissociated subunits with the native enzyme. In order to evaluate reactivity with the native ATPase and the antigenic uniqueness of the five individual subunits of the F_1-ATPase of *M. lysodeikticus*, antibodies have been generated to SDS-β-mercaptoethanol-dissociated subunits excised from SDS-polyacrylamide gel electrophoresis gels, since several reports in the literature have warned against reliance on the use of urea for complete dissociation [24,25]. As reported in this communication we have found that all five SDS-dissociated subunits are immunogenic, antigenically distinct and, moreover, the antibodies react with the native purified F_1-ATPase.

Failure to generate antibodies to all five subunits of the F_1-ATPase may in the past have been due to differences in immunization schedules, including the duration of immunization and individual animal responses to the immunogens. In our experience rabbits responded quite differently to the individual subunit immunogens from purified F_1-ATPase. For example, when the δ-subunit was used as immunogen, good precipitating antibodies were invariably obtained in rabbits within 3–5 weeks after immunization. In contrast to the δ-subunit, it took approx. 10 weeks to obtain precipitating antibodies to the individual α- and β-subunits and at least 3 months for antibodies to the ε-subunit. We failed to obtain strong precipitating antibodies to the γ-subunit in rabbits but the response in guinea pigs was quite good after 9 weeks of immunization. Thus, differences in the individual responses of the same or different animal species as well as intrinsic differences in immunogenicity of the determinants may account for some of the marked discrepancies in the results reported from different laboratories [23–26]. The present investigations with the crossed immunoelectrophoresis method and its variants (e.g., intermediate gel) have permitted a more definitive analysis of the antigenic uniqueness and purity of the individual F_1 subunits and the reactivity of the antisera with the native F_1.

One of the advantages of the crossed immunoelectrophoresis methodology used in our immunochemical study of this F_1 is that immunoprecipitates can be examined for ATPase activity by zymogram staining. This procedure clearly established that only the precipitates formed in the anti-membrane or anti-α- plus β-subunit antisera, after passage through intermediate gels of anti-ε- and/or anti-δ-subunit antibodies, were en-

zymatically active, giving strong ATPase zymogram staining (Figs. 7 and 8). The precipitates formed in the anti-ε- and anti-δ-subunit intermediate zones were inactive and raised the possibility that specific ε- and δ-subunit immunoprecipitates arose from the detachment of these subunits following destabilization of their association with the F_1 on reaction with their respective antibodies. Examination of these immunoprecipitates by SDS-polyacrylamide gel electrophoresis using ^{14}C-labeled F_1 confirmed that most of the δ-subunit was recoverable in the anti-δ-subunit immunoprecipitate, although a small proportion of the total ^{14}C-labeled α- and β-subunits was also detected. The detection of small amounts of α- and β-subunits still associated with the δ-subunits was perhaps not entirely surprising and was compatible with the results of cross-linking experiments of Bragg and Hou [27] and others [28] who have shown α-δ- and β-δ-subunit dimers in bacterial F_1s. The excised immunoprecipitates in the anti-ε-subunit portions of the immunoplates showed low amounts of radioactivity in the ε-subunit regions of the SDS-polyacrylamide gel electrophoresis gels and the control F_1 subunit profiles also exhibited a low level of labeling of the ε-subunit. The reasons for the poor labeling of this subunit are at present unknown. However, it should be pointed out that none of the other labeled subunits were detected in the anti-ε-subunit immunoprecipitate. Moreover, only labeled α- , β- and γ-subunit were detected in the enzymatically active precipitate (in anti-membrane or anti-α- plus β-subunit antisera) after sequential electrophoresis through intermediate gels of anti-ε- and anti-δ-subunit antisera. These results provide strong evidence for the destabilization and detachment of the δ- and ε-subunits from the rest of the F_1 complex (α- , β- , γ-subunits) and further confirm the reconstitution experiments that α- , β- and γ-subunits constitute the enzymatically active (ATPase) complex of the F_1. Destabilization of subunit associations in multimeric proteins reacted with subunit-specific antibodies has been proposed by Bøg-Hansen et al. [29] and our results provide confirmation for this suggestion. Boulain and Menèz [30] have also recently demonstrated that specific antibody binding destabilized the neurotoxin-acetylcholine receptor complex. In addition, it will be recalled that Sternweis [22] used anti-ε-subunit antibodies coupled to Sepharose to remove ε-subunits to yield an α- , β- , γ-subunit complex from a δ-subunit-deficient F_1-ATPase of an *E. coli* mutant.

Dissociation of the δ-subunits prior to interaction with its antibodies appears to be most unlikely, as we would have seen evidence of detachment of free δ-subunit in the first dimension of electrophoresis and its precipitation in a different position in the intermediate gel containing anti-δ-subunit antibodies because of its slower mobility. Moreover, had δ-subunits dissociated from the F_1 they would have been detectable in the upper reference gel in slide b of Fig. 4 after electrophoresis through the intermediate gel containing only anti-α- plus β-subunit antibodies. However, in contrast to this situation with the δ-subunit, there is clear evidence of dissociation of ε-subunits prior to reaction with specific antibodies. During the course of electrophoresis of the F_1 in agarose containing 1% Triton X-100 at pH 8.6 we have observed the progressive detachment of ε-subunits (Figs. 5 and 6). Possible conformational changes occurring during electrophoresis may lead to destabilization of subunit contacts and release of ε-subunits. Indeed, this possibility is strengthened by the interesting results reported by Dreyfus et al. [21] who showed that antibodies to the natural inhibitor protein of bovine heart mitochondrial F_1-ATPase blocks the inhibitory action of the protein on ATP hydrolysis and that ^{121}I-labeled antibodies to the inhibitor bind more readily when the ATPase has been exposed to an electrochemical gradient.

The results of the in vitro inhibition studies presented in Table II show that any antiserum generated against the whole F_1-ATPase molecule (native or denatured) inhibits ATP hydrolysis to the extent of 90–100%. In earlier studies, Whiteside and Salton [31] also showed similar inhibition using antibodies to F_1-ATPase of *M. lysodeikticus* and suggested that inhibition was of a noncompetitive type. The effects of the anti-subunit antibodies on the F_1-ATPase are quite interesting. Present evidence strongly suggests that the β-subunit(s) contain(s) the catalytic portion of the bacterial and chloroplast F_1 and it is therefore easy to envision the inhibitory effect(s) of either anti-β- or anti-α-subunit antiserum. Anti-γ-subunit antiserum has

also been shown to inhibit ATPase activity [23–25]. The inhibitory effects of anti-δ- and anti-ε-subunit antibodies may be explained by the fact that they might be exerting a conformational change within the molecule and thus inhibiting ATPase activity to a lesser extent. Our antisera may also be of a higher titer than other subunit antisera previously used for similar inhibition experiments [23–26]. Recently, Smith and Sternweis [23] have shown that anti-β- and anti-γ-subunit antibodies inhibit ATPase activity in *E. coli*. Although the antibodies to its α- , β- and γ-subunits were obtained by the injection of denatured subunits into rabbits, antibodies to the denatured α-subunits did not react with the native enzyme. Earlier reports also demonstrated that anti-γ-subunit antiserum raised against denatured γ-subunits also inhibited ATPase activity in chloroplasts [24]. Anti-ε-subunit antiserum has also been shown to inhibit membrane-bound ATPase activity in *E. coli* [23].

In conclusion our studies on the detailed immunochemical characterization of *M. lysodeikticus* F_1-ATPase and its subunits have established the antigenic uniqueness of each subunit, the immunogenicity of SDS-dissociated subunits and the reactivity of these specific antibodies with the native molecule of the F_1. Moreover, antibodies generated to the native F_1 contain antibody populations capable of reacting with individual SDS-dissociated subunits. Further investigations would be needed to characterize those antibodies reacting with topographical and/or sequential domains of the subunits. Perhaps one of the most interesting aspects of our investigations raises the possibility of using specific subunit antibodies to destabilize and detach subunits from a multimeric protein, thereby offering a new and novel approach in the study of complex multimeric proteins.

Acknowledgements

This research was supported by Grant Nos. PCM-7824385 and PCM-8105126 from the National Science Foundation. We thank Drs. M.B. Davies and Joel D. Oppenheim for a critical reading of this manuscript, Dr. O.J. Bjerrum for some helpful suggestions, Stan Willard for photography and Michelle Heigh and Josephine Markiewicz for excellent secretarial help.

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