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F_1-ATPase OF *MICROCOCCUS LYSODEIKTICUS* IS NOT A GLYCOPROTEIN

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It has been claimed (Andreu, J.M., Warth, R. and Muñoz, E. (1978) FEBS Lett. 86, 1–5) that the F_1-ATPase of *Micrococcus lysodeikticus* is a glycoprotein containing mannose and glucose as the principal sugars. Even after extensive purification of *M. lysodeikticus* F_1-ATPase by DEAE-Sephadex A25 chromatography, carbohydrate contents varying from 2.7 to 10.8% have been found. Concanavalin A-reactive components corresponding to the succinylated lipomannan have been detected and separated from the ATPase in purified F_1 preparations by immunoelectrophoresis (rocket and two-dimensional) through agarose gels containing concanavalin A. Passage of the purified F_1-ATPase through concanavalin A-Sepharose 4B columns removed the carbohydrate component(s) without loss of the specific activity of the ATPase. Mannose was the only sugar detectable by gas-liquid chromatography of the F_1-ATPase before Con A-Sepharose 4B chromatography and it was completely eliminated after chromatography. No qualitative or quantitative changes in the subunit (α, β, γ, δ and ϵ) profiles were detectable when the sodium dodecyl sulfate polyacrylamide gels were scanned by densitometry of F_1-ATPase before and after Con A-Sepharose 4B chromatography. We conclude that there is no evidence of carbohydrate covalently linked to this F_1-ATPase and that this membrane protein is not a glycoprotein. The presence of carbohydrate is attributable to contamination with lipomannan.

Introduction

It has been suggested by Andreu et al. [1,2] that the energy-transducing F_1-ATPase of *Micrococcus lysodeikticus* is a glycoprotein. Mannose and glucose were the two principal sugars found in the purified F_1-ATPase and smaller quantities of ribose and glucosamine were observed [2]. The claim that coupling factors other than the bacterial F_1 are also glycoproteins was extended to spinach chloroplast F_1 and a complex array of sugars was reported [2]. Moreover, evidence was also presented to support the suggestion that all major subunits of the *M. lysodeikticus* F_1-ATPase were glycosylated [1,3]. These investigators speculated upon the possible linkage of the carbohydrate residues through *O*-glycosidic bonds to the hydroxyamino acids of the peptide moiety but were unable to detect significant β-elimination of serine residues [2].

Previous studies from this laboratory have established that the F_1-ATPase of *M. lysodeikticus* is located on the protoplasmic face of the plasma membrane [4–6] and therefore has an asymmetric distribution as found with other membrane components. In general. it would appear that the hydrophilic glycosylated domains of glycoproteins, glycolipids and lipopolysaccharides are oriented to the external face of membrane and thereby constitute another feature of the asymmetry of biological membranes [7,8]. The suggestion that a major glycoprotein such as the F_1-ATPase occupies an internal orientation towards the cytosol compartment of the cell raises some basic questions about membrane asymmetry and architecture. Since, to our knowledge, the claim of the glycoprotein nature of a bacterial F_1-ATPase (or

Abbreviations: SDS, sodium dodecyl sulfate; PMSF, phenylmethylsulfonyl fluoride.

other F_1 types for that matter) has not been independently substantiated, we decided to seek further evidence for the covalent association of carbohydrate residues with the F_1-ATPase purified under our conditions which yield a classical five-subunit (α, β, γ, δ and ϵ) F_1 [9].

Materials and Methods

Purification of F_1-ATPase. *M. lysodeikticus* (NCTC 2665) was grown in peptone/water/yeast extract medium and cells were harvested as described previously [10]. Purification of F_1-ATPase was performed according to the method of Huberman and Salton [9] except that PMSF was omitted in the shock-wash step. The purified ATPase was dialyzed against 50 mM Tris-HCl (pH 7.5) containing 20% glycerol and stored in this buffer at 4°C, and under these conditions no degradation of the subunits was detected during the course of the experiments. Omission of PMSF as previously described [9] was based on recent observations that stability, latency and subunits of the F_1-ATPase were well preserved in the presence of 20% glycerol for short periods (about 1 week) of storage at 4°C. However for longer periods of storage of a month's duration, addition of PMSF was necessary (Urban, C., personal communication).

Con A-Sepharose 4B column chromatography. A column (1.3 × 10 cm) of Con A-Sepharose 4B (Pharmacia Fine Chemicals Inc., Piscataway, NJ) was equlibrated at 4°C with a 40-times bed volume of 50 mM Tris-HCl (pH 7.6) containing 1 mM $MnCl_2$ and 1 mM $CaCl_2$ to remove the loosely bound subunits of concanavalin A. Both divalent metal ions are necessary for maintenance of concanavalin A-binding ability and reproducible results [11]. 12–14 mg of the purified ATPase preparations were applied and the column was eluted with the Tris-HCl/Mn^{2+}/Ca^{2+} buffer. Fractions of 10.5 ml were collected at an approximate rate of 1 ml/min. Absorbance was measured at 280 nm. Eluted fractions were assayed for ATPase activity with and without trypsin treatment according to the method of Huberman and Salton [9].

Immunoelectrophoretic techniques. Two-dimensional (crossed) immunoelectrophoresis and rocket immunoelectrophoresis with intermediate gels containing concanavalin A were performed under the conditions and by the techniques previously described in detail [5,6]. Antisera to *M. lysodeikticus* membranes were generated in New Zealand white rabbits following the immunization schedules previously used in this laboratory [12]. Immunoglobulin fractions were obtained by the method of Harboe and Ingild [13] and concentrated 5-fold with respect to original serum volumes. Reference gels contained 0.3 ml of the anti-membrane immunoglobulins in a total of 2 ml of agarose and the intermediate gels contained 0.2 mg/ml of concanavalin A with $MnCl_2$ (50 μM) and $CaCl_2$ (50 μM). Rocket electrophoresis was performed at 2.5 V/cm at 10°C for 14–16 h.

Gels were soaked in 0.1 M NaCl overnight and the process of soaking for 50 min in 0.1 M NaCl and pressing for 5 min was repeated six times. Gels were dried under warm air and stained with 0.25% Coomassie brilliant blue dissolved in ethanol/water/acetic acid (9 : 9 : 2, v/v/v).

Gas-liquid chromatography. F_1-ATPase preparations obtained before and after passage through the Con A-Sepharose column were dialyzed against distilled H_2O for two 12 h periods at 4°C to remove the Tris which would otherwise interfere with the gas chromatography. The dialyzed fractions were lyophilized and 2 mg of each ATPase fraction were hydrolyzed with 2 ml of 0.1 M trifluoroacetic acid in a sealed tube at 120°C for 6 h to liberate any sugars present [14]. These conditions were found to give close to maximal liberation of monosaccharide by carrying out a hydrolysis curve with an ATPase preparation containing carbohydrate. The acid hydrolysates were cooled in ice and adjusted to pH 8.0–8.5 with NaOH. The sugars obtained from hydrolyzed polysaccharides were reduced to their alditols with $NaBH_4$ (20 mg) at 24°C for 1 h and excess $NaBH_4$ was decomposed by the addition of glacial acetic acid and the solution was evaporated almost to dryness at 85°C under reduced pressure [14]. Approx. 3 ml of methanol/HCl (100 : 0.1, v/v) were added and heated for 5 min at 85°C and the solution was evaporated [15]. This step was repeated seven times to remove borate and prevent retardation of acetylation [14,15] and the dried samples were stored overnight in a vacuum desiccator containing P_2O_5 at room temperature. Acetylation of alditols was carried out with the addition of 2 ml of acetic anhydride to the dried sample at 120°C for 2 h and

the residual anhydride was evaporated under a stream of N_2 gas while the solution was still hot [14]. Acetylated alditols were extracted with 3.0, 2.0 and 1.5 ml of methylene dichloride consecutively. Inorganic materials were removed by centrifugation at 5000 rev./min for 10 min and 20 μl of the samples dissolved in methanol were injected into a F & M Model 400 Biomedical gas chromatograph equipped with a flame ionization detector. A 6 ft × 2 mm inner diameter glass column packed with 3% SP-2330 on 100/120, Supelcoport (Supelco, Inc., Bellefonte, PA) was used and the conditions of operation were as follows: column temperature 205°C, carrier gas (N_2) at a flow rate of 20 ml/min and temperature of injection port and detector was 270°C.

Analytical procedures. Carbohydrate contents of the lyophilized ATPase preparations were determined by the colorimetric anthrone method of Morris [16], using mannose as the standard, since it was suspected that the carbohydrate may be contaminating lipomannan. Protein was determined by the method of Lowry et al. [17] with bovine serum albumin as standard.

Polyacrylamide gel electrophoresis. For SDS-polyacrylamide gel electrophoresis, 10% polyacrylamide slab gels containing 0.1% SDS in Tris-glycine buffer (pH 8.6) were prepared according to the method of Laemmli [18]. ATPase preparations obtained before and after Con A-Sepharose 4B chromatography were denatured by boiling for 1 min and applied to the slab gel. A constant current of 10 mA was applied for 1.5 h, followed by 20 mA for 3 h. Electrophoresis was performed at room temperature. Gels were stained for protein with Coomassie brilliant blue and destained with methanol/water/acetic acid (9 : 9 : 2, v/v/v) for 1 h and stored in 7% acetic acid. The protein bands were scanned with Microdensitometer 3 CS (Joyce Loebl) from negative films of the gels.

Chemicals. Seakam HGT agarose was obtained from Marine Colloids, Inc. (Rockland, ME). Acetic anhydride (at least 99%) and methyl alcohol (99.9%, spectrophotometric grade, Gold label) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Methylene dichloride was purchased from Sigma Chemical Co. (St. Louis, MO); D-(+)-mannose was from Fisher Scientific Co. (Springfield, NJ); concanavalin A was from Miles Lab. Inc. (Elkhart, IN); anthrone was obtained from Matheson Coleman and Bell (East Rutherford, NJ) and all other chemicals were analytical grade from commercial sources.

Results

Although previous studies had established the presence of succinylated lipomannan in membrane extracts by crossed (two-dimensional) immunoelectrophoresis and rocket electrophoresis into membrane antibodies and/or concanavalin A [5,12,19], F_1-ATPase purified by chromatography on DEAE Sephadex A25 [9] had not been examined for the presence of carbohydrate components. Accordingly, fractions of F_1-ATPase purified to near protein homogeneity by the method of Huberman and Salton [9] were subjected to analysis by crossed immunoelectrophoresis with concanavalin A in the intermediate gel [5]. The results presented in Fig. 1 show the electrophoretic separation of the F_1-ATPase from material forming precipitates with concanavalin A in the intermediate gel. The two precipitates seen in the concanavalin A intermediate gel are probably due to charge heterogeneity of the succinylated lipomannan, a phenomenon previously observed with this membrane amphiphile [5,6,19]. In confirmation of previous studies [5], the ATPase immunoprecipitates were unaffected by electrophoresis through concanavalin A (cf. A and B of Fig. 1). These results suggested that the concanavalin A-reactive materials in the purified F_1-ATPase were not covalently linked to this protein.

Concanavalin A-Sepharose 4B chromatography of purified F_1-ATPase

In order to remove concanavalin A-reactive components, the purified F_1-ATPase was subjected to Con A-Sepharose chromatography as described in Materials and Methods. It should be noted that in preliminary experiments there was evidence of coelution of the ATPase with concanavalin A subunits stripped from the column. Accordingly, the Con A-Sepharose column was extensively washed with elution buffer containing Mn^{2+} and Ca^{2+} [11] prior to chromatography of the F_1-ATPase preparations. The results of a typical experiment are illustrated in Fig. 2, showing the elution profile of protein (280 nm absorbance) and ATPase activity assayed in the pres-

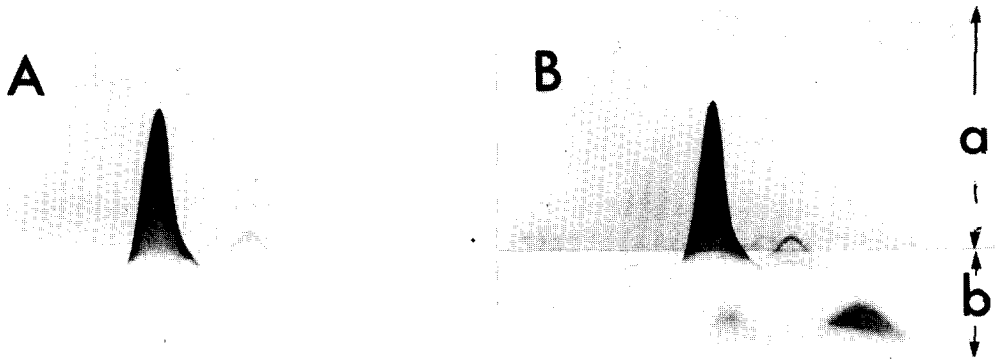

Fig. 1. Crossed, two-dimensional immunoelectrophoresis of F_1-ATPase with intermediate gel containing concanavalin A. In immunoplates A and B, 3 μg of F_1-ATPase (containing 10.8% carbohydrate by anthrone method) purified by DEAE-Sephadex chromatography were electrophoresed at 8.9 V/cm for 1 h at 10°C in the first dimension. Electrophoresis in the second dimension was performed at 2.5 V/cm for 16 h at 10°C through an intermediate gel (zone b) containing only buffered agarose (plate A) or agarose with concanavalin A (0.2 mg/ml), 50 μM Mn^{2+}, 50 μM Ca^{2+} (plate B), into the reference gels (zone a) containing 0.3 ml of antimembrane immunoglobulins/2 ml agarose. Note the formation of precipitates in the intermediate gel (plate B) containing concanavalin A and identical peaks of ATPase immunoprecipitates in both plates A and B. Several minor immunoprecipitates due to trace impurities in the preparation are seen in the reference gels. Anodal migration is to the left and top of each plate.

ence and absence of trypsin [9]. The trypsin-stimulated activity of this latent ATPase was 3.3–3.5-fold higher for each eluted fraction than the activity (basal) found for the untreated (no trypsin) fractions. The tailing of the peak seen in Fig. 2 may be attributable to nonspecific hydrophobic interactions with the Con A-Sepharose. Recovery of protein [17] was determined for three different preparations of F_1-ATPase on pooled fractions 1–8 following Con A-Sepharose chromatography and an average value of 82% was obtained.

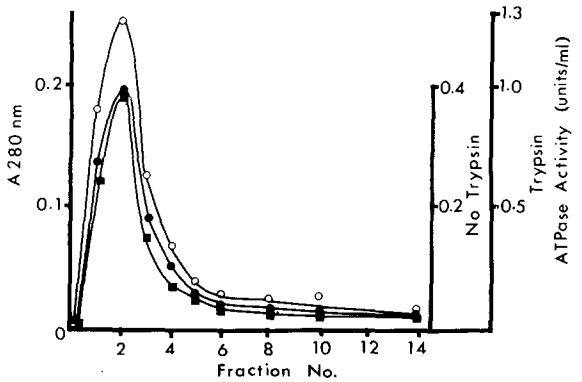

Fig. 2. Affinity chromatography of purified F_1-ATPase on Con A-Sepharose 4B. 13.0 mg purified F_1-ATPase were applied to the column and eluted with 50 mM Tris-HCl buffer (pH 7.5) containing 1 mM $MnCl_2$ and 1 mM $CaCl_2$ as described in Materials and Methods. 10.5-ml fractions were collected and analyzed for absorbance at 280 nm (■———■) and ATPase activity in the presence (○———○) and absence (●———●) of trypsin as described in Materials and Methods.

Fractions from the Con A-Sepharose column were examined by rocket immunoelectrophoresis with an intermediate gel containing concanavalin A to determine the amount of ATPase (as antigen) and to detect the presence of any carbohydrate precipitable with concanavalin A. In the results illustrated in Fig. 3, the reference gel (a) contained antibodies to membrane and concanavalin A was added to the intermediate gel (b) as described in Materials and Methods. As shown very clearly in Fig. 3, the F_1-ATPase contained carbohydrate before passage through the Con A-Sepharose column, but no precipitates were detectable in the fractions obtained after Con A-Sepharose chromatography. This procedure has thus removed the carbohydrate component previously identified as the succinylated lipomannan [5,6]. The recovery of ATPase determined by quantitative rocket immunoelectrophoresis as illustrated in Fig. 3 averaged 79% (for three preparations), a value in good agreement with the recovery of protein determined by the method of Lowry et al. [17].

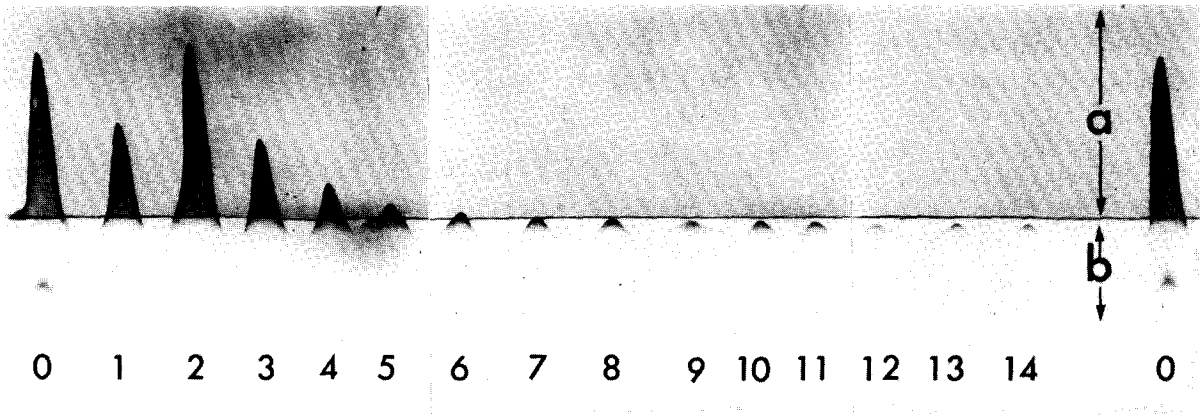

Fig. 3. Rocket immunoelectrophoresis through an intermediate gel (zone b) containing concanavalin A (as for Fig. 1) of the fractions eluted from the chromatography of F_1-ATPase on Con A-Sepharose 4B (as for Fig. 2). Well 0 contained 3.1 μg of the DEAE-Sephadex purified F_1-ATPase before removal of carbohydrate by affinity chromatography. Wells 1–14 contained 8 μl of each fraction (10.5 ml) eluted from the Con A-Sepharose column. Zone a of the immunoplates contained anti-membrane immunoglobulins as for Fig. 1. Note the presence of precipitates in the intermediate gel containing concanavalin A of the F_1-ATPase preparation before chromatography on Con A-Sepharose and the absence of precipitates after removal of carbohydrate. Anodal migration was to the top of the plates.

Gas-liquid chromatography

In order to identify the sugars of the carbohydrate present in the purified F_1-ATPase before and after removal of carbohydrate by Con A-Sepharose chromatography, gas-liquid chromatography was employed. Sugars were identified as alditol acetates following hydrolysis of the preparations with 0.1 M trifluoroacetic acid as described in Materials and Methods. As shown in Fig. 4A, F_1-ATPase which was purified by DEAE-Sephadex A25 chromatography gave a single peak with a retention time of 8.6 min on gas-liquid chromatography. This sugar has an identical retention time to that of authentic alditol acetate of mannose and comigrated when the latter was used as the internal standard. However, this sugar peak (mannose) was totally eliminated from the F_1-ATPase after passage through the Con A-Sepharose column and no other sugars were detectable (Fig. 4B). Andreu et al. [1] reported that the sugars in their F_1-ATPase were mainly mannose and glucose and smaller quantities of other sugars. Had these been present in our preparations they should have been readily detectable and identifiable.

Analysis of F_1-ATPase preparations before and after Con A-Sepharose 4B chromatography

In order to compare the properties of the ATPase before and after Con A-Sepharose chromatography under conditions of average recoveries of approx. 80% protein, the specific activities and carbohydrate

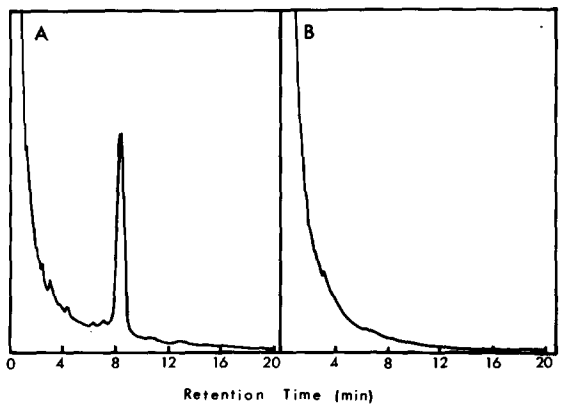

Fig. 4. Gas-liquid chromatographic analysis of purified F_1-ATPase before (A) and after (B) chromatography on Con A-Sepharose 4B. Fractions were hydrolyzed with trifluoroacetic acid and sugars converted to alditol acetates for gas-liquid chromatography as described in Materials and Methods. Samples injected correspond to 160 μg dry weight of original ATPase preparations before and after affinity chromatography. The single major peak found before affinity chromatography (A) was identified as mannose and it was completely eliminated from the F_1-ATPase preparation after Con A-Sepharose chromatography (B).

contents were determined. For assaying ATPase activity, fractions 1–8 were concentrated and dialyzed overnight at 4°C against 50 mM Tris-HCl (pH 7.5) containing 20% glycerol. Specific activities and carbohydrate contents determined by the anthrone and gas-liquid chromatographic methods are summarized in Table I. As shown in Table I, the basal, low intrinsic ATPase activity of this F_1 (no trypsin) was unaltered by passage through the Con A-Sepharose column but the fraction obtained after removal of carbohydrate showed higher levels of trypsin stimulation. The reason for the increase in latency cannot be fully explained at the present time but could be due to removal of noncovalently bound lipomannan or contaminating proteins, a portion of which may have blocked the catalytic sites of the ATPase. Nonspecific protein-binding capabilities of bacterial amphiphiles such as the lipoteichoic acids have been well established [20] and the coseparation of lipomannan and F_1-ATPase we have observed suggests that it behaves similarly.

Quantitative determination of carbohydrate by the anthrone method gave a batch-to-batch range of 2.7–10.8% for the F_1-ATPase purified by DEAE Sephadex chromatography [9] and after removal of carbohydrate by Con A-Sepharose chromatography the anthrone reaction gave values of 0.3–0.5%. These low values could be due to nonspecific colored products formed under the conditions of the anthrone method, since no sugars were detectable by gas-liquid chromatography. Calculation of the mannose content from the alditol acetate peaks on the gas-liquid chromatograms gave values in good agreement with those estimated by the anthrone method (Table I).

TABLE I

SPECIFIC ACTIVITIES AND CARBOHYDRATE CONTENTS OF F_1-ATPase PREPARATIONS BEFORE AND AFTER CON A-SEPHAROSE 4B CHROMATOGRAPHY

	Before	After
Specific activity [a]		
–Trypsin	2.09	2.45
+Trypsin	5.65	6.68
% carbohydrate [b]		
Anthrone	2.7, 3.4, 10.8	0.3, 0.5, 0.4
GLC	n.d. 3.0, 8.3	none detectable

[a] Mean values of specific activities (μmol P_i/min per mg; 37°C) for three different preparations.

[b] Carbohydrate content determined as % (w/w) by anthrone method and by gas-liquid chromatigraphy (GLC). n.d. not done.

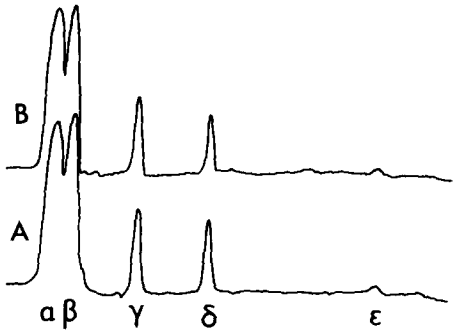

Fig. 5. SDS-polyacrylamide gel electrophoresis of F_1-ATPase before (scan A) and after (scan B) chromatography on Con A-Sepharose 4B. Gels prepared and scanned as described in Materials and Methods. 54 μg protein of each preparation of the F_1-ATPase before and after affinity chromatography were run side by side on the slab gel. The subunits are indicated by Greek letters. Top of the gel to the left and dye front to the right.

Polyacrylamide gel electrophoresis

Since the F_1-ATPase of *M. lysodeikticus* is a multimeric protein possessing five subunits, it was important to establish whether any subunits which may be glycosylated were selectively removed on passage through the Con A-Sepharose. Accordingly, the F_1-ATPase subunit profiles were examined before and after Con A-Sepharose chromatography. Slab gels were stained with Coomassie brilliant blue, photographed and the negatives scanned in a Joyce Loebl densitometer. As shown in Fig. 5A and B, all five subunits were present and, moreover, in approximately the same proportions after removal of the carbohydrate. There is, therefore, no evidence of change in subunit composition nor in the relative proportions of the subunits following carbohydrate removal that could be correlated with glycosylation of F_1-ATPase subunits.

Discussion

It is evident from the studies reported in this communication that *M. lysodeikticus* F_1-ATPase can

be purified to near protein homogeneity by chromatography on DEAE Sephadex A25 [9] yet still contain variable and significant amounts of contaminating membrane succinylated lipomannan. The latter component would not be detected on SDS-polyacrylamide gel electrophoresis of F_1-ATPase subunits stained for protein with Coomassie brilliant blue, a criterion often used for establishing protein homogeneity. However, we have shown that components reacting with concanavalin A can be separated physically from the F_1-ATPase by electrophoresis (see Figs. 1 and 3) in agarose gels by the sensitive, high-resolution, crossed immunoelectrophoresis and rocket electrophoresis techniques. The presence of concanavalin A -reactive mannose and/or glucose residues on the F_1-ATPase protein would have manifested itself by retardation of the migration of the F_1 through intermediate gels or by precipitation in the gel containing concanavalin A. In confirmation of our previous studies [5,6] this was not observed. These results, however, did not rule out the possibility that these sugars were present but in configurations unreactive with concanavalin A. Furthermore, the possibility existed that F_1-ATPase polypeptides were glycosylated with other sugars. Gas-liquid chromatography was accordingly used to identify the sugar component and only one sugar, mannose, was detected in the F_1-ATPase purified by DEAE Sephadex chromatography. After removal of carbohydrate by Con A-Sepharose chromatography under conditions of 80% recovery of protein and retention of ATPase specific activity, the mannose was completely eliminated and no other sugars were detectable. Moreover, no change in the subunit composition, relative proportions of the subunits or changes in molecular weights (due to proteolytic cleavage of subunits) following removal of the contaminating carbohydrate component(s) could be detected. Thus, we could find no evidence to support the claim of Andreu et al. [1,2] and Guerrero et al. [3] that *M. lysodeikticus* F_1-ATPase is a glycoprotein. The total absence of detectable sugars after Con A-Sepharose chromatography and recovery of intact F_1-ATPase leads us to the conclusion that this ATPase is not a glycoprotein. Contamination of preparations studied by Andreu et al. [1,2] with lipomannan could in part account for their conclusions but at the present time there is no obvious explanation for the presence of the other sugar components (e.g., glucose, ribose, glucosamine) reported in their preparations. Our results emphasize the need for rigorous exclusion of contaminating molecular species such as the lipomannan from membranes and reinforces the utility of immunoelectrophoresis and affinoelectrophoresis techniques in establishing homogeneity of purified membrane components.

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