ISOLATION AND PARTIAL CHARACTERIZATION OF THE TWO MAJOR SUBUNITS OF THE BF_1 FACTOR (ATPase) FROM *MICROCOCCUS LYSODEIKTICUS* AND EVIDENCE FOR THEIR GLYCOPROTEIN NATURE

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1. Introduction

Purified bacterial ATPases (BF_1 coupling factors) resemble the soluble coupling factors, F_1 and CF_1, of mitochondria and chloroplasts [1–3] by their mol. wt. [4–7], amino acid composition [4,6] and presence of two major subunits (α and β) of mol. wt. about 55 000 [4–9]. The isolation and characterization of the subunits of different ATPase systems may help in further elucidating the functional and evolutionary significance of these energy-transducing proteins. The subunits of mitochondrial F_1 ATPase were isolated in preparative scale and their molecular weights and amino acid compositions determined [10]. Nelson et al. [11] isolated limited amounts of the CF_1 subunits, characterized them chemically and prepared antisera against the isolated subunits. Recently Kanner et al. [12] prepared antisera against the three major subunits of *Escherichia coli* ATPase. In this paper, we describe for the first time the isolation in preparative scale of the two major subunits (α and β) of a bacterial F_1 factor and their partial characterization. We also present evidence suggesting the glycoprotein nature of the ATPase of *M. lysodeikticus* and their α and β subunits and discuss it in relationship to the micro-heterogeneity and sensitivity to alkali of this BF_1 factor [13,14].

Abbreviations: PAGE, polyacrylamide gel electrophoresis; PAS, periodic acid–Schiff stain; Gu-HCl, guanidine hydrochloride.

2. Materials and methods

2.1. *Gel electrophoresis*

Polyacrylamide gel electrophoresis was performed on 100 × 6 mm gels (analytical) or 40 × 50 mm gels (preparative), polymerized for 1 h at room temperature in 0.2 M imidazole (Merck) -HCl (pH 7.2) with 0.05% (w/w) ammonium persulphate and 0.08% (v/v) *N,N,N',N'*-tetramethylethylendiamine (Fluka). The ratio acrylamide: *N,N'*-methylenebisacrylamide (Eastman Kodak) was kept constant at 38:1. Upper and lower chamber buffers were, respectively, 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethane-sulphonic acid (Calbiochem) -imidazole (pH 7.0) [15] and 0.1 M imidazole-HCl (pH 7.0). In some instances, a Tris-glycine/Tris-HCl buffer system similar to that developed before [16] but at pH 8.0 was used. Electrophoreses were performed at 150 V with Bromophenol blue as tracking dye. The actual pH values at the end of the electrophoretic runs were measured in eluted gels and buffers and amounted to 7.4 ± 0.2 for the imidazole system and to 8.4 ± 0.4 for the pH 8.0 system. Analytical gels were stained with Coomassie R250 [17] and the periodic acid–Schiff reaction [18] for carbohydrate, and scanned at 565 nm (Coomassie) and 575 nm (PAS) using a Gilford 2400 equipped with the model 2410-S linear transport. Lysozyme (Calbiochem), soybean trypsin inhibitor (Boehringer), trypsin (Calbiochem), egg albumin (Sigma), aldolase (Sigma), catalase (Calbiochem), bovine serum albumin (Sigma) and urease (Calbiochem) were used as standards for molecular weight determinations and staining procedures.

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2.2. *Chemical analyses*

Amino acid analyses of the purified ATPase and subunits were carried out from hydrolysates of 20 μg protein with 6 N HCl at 110°C for 22 h in a Durrum D-500 amino acid analyzer. Hexosamines were determined by ion exchange chromatography in a Jeol JLC-5AH autoanalyzer, following the purification of protein hydrolysates (3 N HCl, 110°C 6 h) as described [19].

Hexoses were estimated by the sulphuric acid–cysteine reaction [20] and the phenol–sulphuric acid method [21], scaled down to allow the determination of 5–10 μg and 0.5–2 μg neutral sugar, respectively. The orcinol reaction [22] brought to microscale allowed the detection of 1–2 μg of galactose-mannose. Appropriate blanks of Diaflo ultrafiltrates and buffer concentrates of each fraction were run to avoid any artifactual interference. In some instances, protein samples were precipitated with ethanol before the assays for neutral sugar.

3. Results

3.1. *Purification of α and β subunits and their characterization by polyacrylamide gel electrophoresis*

Fig.1 shows the elution profile of a preparative run in the 8 M urea–PAGE system with two peaks corresponding to the α and β subunits of *M. lysodeikticus* ATPase. Each peak was subsequently examined by urea and sodium dodecyl sulphate analytical gel electrophoresis at different pH values and acrylamide concentrations. This analysis demonstrated that the isolated subunits were more than 90% pure as judged by protein staining. Fig.2 illustrates some examples. Mol. wts. of 58 000 and 55 000 dalton were calculated for the isolated α and β subunits from their mobilities in the dodecyl sulphate–PAGE system with minor variations at different gel concentrations [23]. Some mobility data of the subunits in the 8 M urea–PAGE system at different gel concentrations analyzed by the retardation coefficient versus molecular size procedure [24] lead to calculated mol. wts. of 50 000 ± 10 000 for α and β with a small difference of 1000–2000 between them.

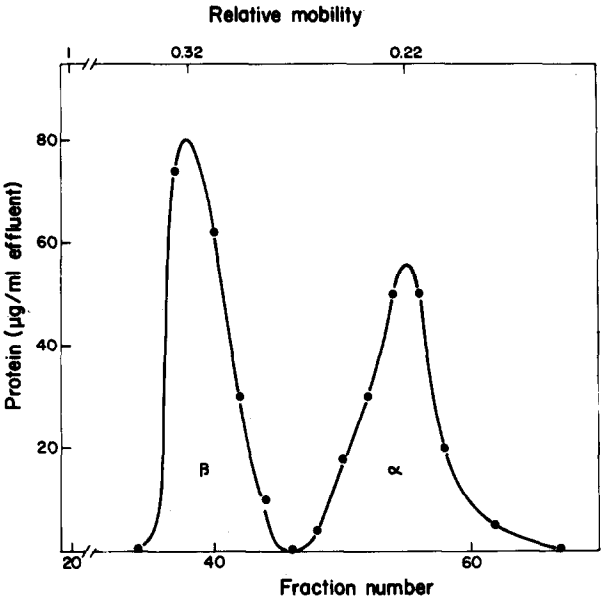

Fig.1. Isolation of α and β subunits of *M. lysodeikticus* ATPase by preparative 6% polyacrylamide gel electrophoresis in 8 M urea at pH 7.2. The upper buffer and gel (see Materials and methods) were added of 8 M urea (Merck, biochemical use) – 1 mM dithiothreitol (Calbiochem). Approximately 10 mg of form A of ATPase, purified by preparative gel electrophoresis as described [15] but using the imidazole-HCl (pH 7.0) buffer system, were pretreated with 10 M urea–5 mM dithiothreitol and applied to the gel. Effluent (fractions of 8 ml were collected each 24 min) was monitored by absorbance at 206 nm in an Uvicord III (LKB). The protein contents were estimated by comparing analytical gels in dodecyl sulphate- and urea–PAGE with known amounts of whole ATPase dissociated in the same conditions as standards. The peaks were concentrated and transferred to 50 mM Tris-HCl (pH 7.5)–1 mM dithiothreitol by continuous ultrafiltration through an extensively washed Diaflo PM 10 membrane and then stored at –20°C. For other details see the text.

3.2. *Lability to pH and microheterogeneity of α subunit*

When α subunit was prepared in the standard Tris-HCl buffer system (actual pH 8.4 ± 0.4) from an ATPase purified under the same conditions, we obtained a microheterogenous preparation. It consisted of 4–5 closely running bands in the analytical 8 M urea–PAGE system (fig.2d) and of, at least, two components in dodecyl sulphate gel electrophoresis (see fig.2 h). The first of these two components had the same mobility as α whereas the

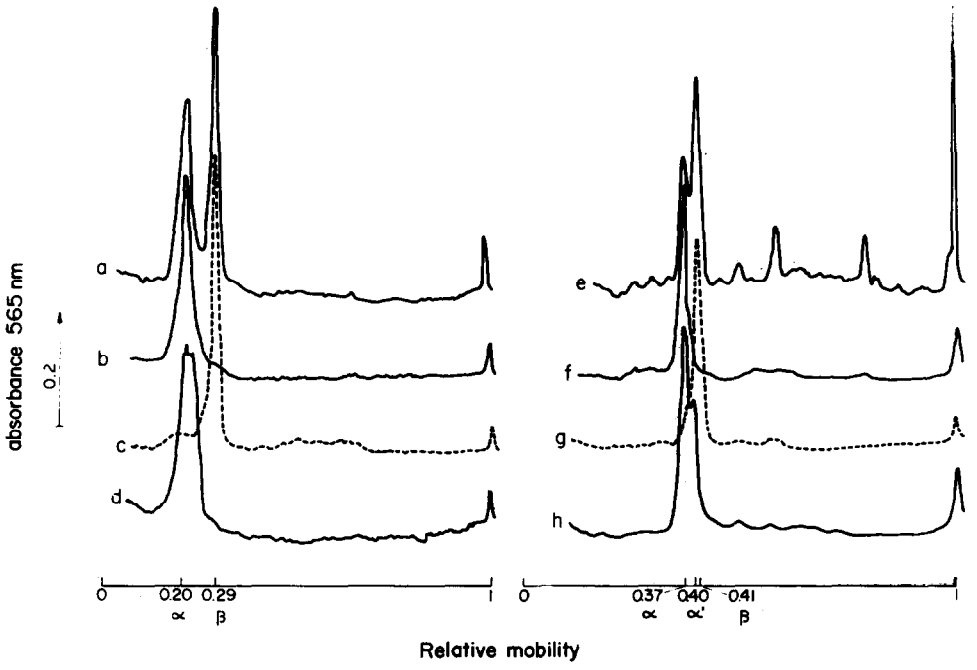

Fig.2

second one, α', ran ahead of α and close to β. When four different samples containing: (1) isolated and structurally intact α subunit; (2) a mixture of α and β subunits; (3) denaturated ATPase in either urea or sodium dodecyl sulphate; and (4) native ATPase, were exposed to pH values ranging from 8 to 11 during 1 h at 17°C at a protein concentration of 30–40 μg of each subunit per ml, similar transformations of α were observed. The extent of its degradation was dependent on the treatment and followed the order $\alpha \leqslant \alpha + \beta <$ denatured ATPase < native ATPase. Storage at −20°C at pH 8.4 of α subunit in 8 M urea also induced a relative increase in the proportion of α' when compared to preparations kept in 6 M Gu-HCl (pH 7.5) at the same temperature.

3.3. *Chemical composition of* M. lysodeikticus BF_1 *and* α *and* β *subunits*

Fig.3 shows the positive PAS stain of the native

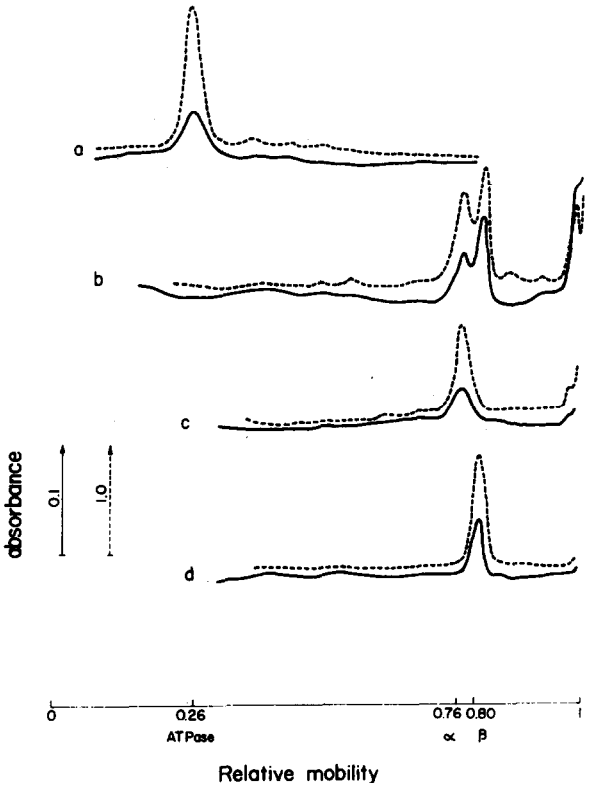

Fig.3. Carbohydrate stain (solid line) and the corresponding protein stain (broken line) of *M. lysodeikticus* ATPase and its α and β subunits. (a) Native ATPase (10 μg) electrophoresed in 7% acrylamide at pH 8.0. Electrophoresis in 6.5% sodium dodecyl sulphate–PAGE at pH 8.0: (b) whole ATPase (20 μg); (c) 6 μg of α subunit; (d) 6 μg of β subunit. For other details see text and legend of fig.2.

Fig.2. Analytical gel electrophoresis of *M. lysodeikticus* ATPase and its α and β subunits. (Left) urea–PAGE at pH 7.2 and 6% acrylamide (for experimental details see the text and legend of fig.1): (a) whole ATPase; (b) subunit α; (c) subunit β; and (d) subunit α prepared at pH 8.0 (see text). (Right) dodecyl sulphate–PAGE at pH 7.2 and 10% acrylamide. Specially pure sodium dodecyl sulphate (BDH) at 0.1% (w/v) was incorporated in buffers and gels. Prior to electrophoresis protein samples were treated with a 10-fold excess by weight of sodium dodecyl sulphate. (e) Whole ATPase; (f) α; (g) β and (h) subunit α prepared at pH 8.0. All samples contained approximately 6 μg of each subunit. Migration was from left to right.

Table 1

Chemical composition of *M. lysodeikticus* ATPase and of its α and β subunits as compared with those of two organelle ATPase

	M. lysodeikticus (mol/100 mol)			Chloroplast			Mitochondria		
Amino acids	α	β	BF_1	α	β	CF_1	1	2	F_1
Lys	3.58	2.98	3.81	3.77	4.06	4.38	6.35	5.23	6.09
His	1.96	1.59	1.81	0.56	0.99	0.80	1.19	1.74	1.43
Arg	7.43	5.27	6.64	5.71	5.84	6.37	5.36	4.14	5.38
Asp	9.43	11.65	9.64	7.15	8.65	7.57	8.33	7.63	7.88
Thr	5.57	7.78	6.61	6.75	7.25	7.17	4.96	5.88	5.38
Ser	7.03	6.63	5.02	6.23	5.98	5.98	7.34	5.23	5.02
Glu	13.86	12.12	12.10	15.28	11.50	13.94	11.51	12.64	12.54
Pro	2.76	3.13	2.97	3.23	5.19	3.98	3.57	5.23	4.30
Gly	10.32	10.09	10.22	8.04	9.64	8.37	11.31	10.02	9.68
Ala	9.61	8.66	11.27	10.86	8.51	9.16	9.92	10.46	10.04
Cys[a]	–	–	–	0.34	0.61	0.40	0.60	0.22	0.36
Val	7.75	8.38	8.52	7.03	8.14	7.17	7.94	8.72	8.24
Met	1.77	1.64	1.54	2.11	2.75	2.79	1.59	1.96	1.79
Ile	5.73	5.67	5.10	7.37	5.35	6.77	6.94	6.54	7.17
Leu	9.40	8.86	9.80	9.87	9.94	9.56	8.93	8.93	9.32
Tyr	1.15	1.94	2.20	3.28	2.44	2.97	1.79	2.40	2.51
Phe	2.61	3.63	2.77	2.46	3.23	2.79	2.38	3.05	2.87
Asp + Glu (acid) / Lys + Arg + His (basic)	1.79	2.44	1.79	2.22	1.89	1.85	1.54	1.82	1.58
Carbohydrate	g/g peptide								
Hexoses									
(i) orcinol	0.12	0.6	0.12						
(ii) cysteine sulphuric			0.15						
(iii) phenol sulphuric			0.19						
Hexosamines									
glucosamine			0.03						
galactosamine			0						

[a] Cysteine was not measured. We knew from previous work [6] that *M. lysodeikticus* BF_1 had a very low amount.

The amino acid and carbohydrate analyses were carried out as described in Materials and methods. Data of α and β chloroplast subunits were taken from the work by Nelson et al. [11], of chloroplast coupling factor (CF_1) from Farron [3] and of subunits 1 and 2 and coupling factor F_1 from Knowles and Penefsky [10].

ATPase (a) and most of the components of the dodecylsulphate-dissociated ATPase (scan b) as well as of the isolated subunits (scans c and d). The value of the method was assessed by the positive results obtained with standard glycoproteins (e.g. egg albumin) and the negative results with trypsin, lysozyme and bovine serum albumin. The glycoprotein nature of *M. lysodeikticus* ATPase and of their major subunits was confirmed by their hexose contents and by the presence of hexosamines as shown in table 1. Subunit α seems to have twice the amount of neutral sugars of β.

The amino acid composition as reported in table 1 shows that α and β subunits of *M. lysodeikticus* . ATPase resemble those of their homologous mitochondrial and chloroplast ATPases. Subunit α differs from β in its higher ratio basic/acid amino acids which justifies its lower mobility in the 8 M urea–PAGE system [16] and in its lower contents of aromatic amino acids.

4. Discussion

This work describes for the first time the isolation in a preparative scale by a single step procedure of the two major subunits of a bacterial ATPase or BF_1 factor.

The glycoprotein nature of this bacterial ATPase is a novel feature of coupling factors. It is worth noting that Guidotti stated recently that all membrane transport processes would be catalyzed by oligomeric glycoproteins which span the membrane [25]. Our result may extend this statement to bacterial membrane ATPases, although, to the best of our knowledge, the BF_1 factors are not transmembrane proteins [26]. The role of sugars in *M. lysodeikticus* ATPase is open to speculation. It is tempting to postulate that the sugars may play a role in divalent cation binding or be involved in the conformational changes and polypeptide modifications during ATP hydrolysis ([27] and Carreira, J. and Muñoz, E., unpublished observations). Preliminary experiments carried out by O. Azocar in this laboratory showed that *E. coli* ATPase also had a positive PAS stain and that it was partially inhibited by concanavalin A. The glycoprotein nature of BF_1 factors might be a differential characteristic with regard to mitochondrial and chloroplast ATPase since all the hexosamine analysis of the organelle coupling factors gave negative results [1,2].

The lability of α to alkaline pH was reminiscent of the general sensitivity to alkali shown by the whole enzyme [13,14] and may be responsible for its constitutive microheterogeneity [13]. It could be justified by its sugar contents.

The mol. wt. of the isolated subunits must be considered as tentative. Although the behaviour of the α and β subunits in dodecyl sulphate-PAGE was almost normal (see above), one cannot forget that glycoproteins interact anomalously with dodecyl sulphate [23]. One of the most serious approaches to the determination of their mol. wt. involves the application of high speed equilibrium sedimentation [10] but speeds enough to achieve a good meniscus depletion at solution densities of about 1.14 [10] and the actual values of the partial specific volume in presence of Gu-HCl would be required. On the other hand, the presence of small mol. wt. species may induce gross errors in the estimation of mol. wts. by this method ([28] and Andreu, J. M., unpublished observations). Some years ago, Schnebli et al. determined a mol. wt. of 30 000 for the Gu-HCl-dissociated ATPase of *Streptococcus faecalis* [29]. A detailed analysis of the size (e.g. low speed equilibrium) and chemical composition of α and β subunits is now in progress.

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