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MICROCOCCUS LYSODEIKTICUS ATPase

PURIFICATION BY PREPARATIVE GEL ELECTROPHORESIS AND SUBUNIT STRUCTURE STUDIED BY UREA AND SODIUM DODECYLSULFATE GEL ELECTROPHORESIS

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SUMMARY

Micrococcus lysodeikticus ATPase was purified by preparative gel electrophoresis after its "shock wash" release from the membrane. The method afforded the highest yield of pure protein in the minimum time as compared with former purification procedures. The pure protein had a specific activity of 7 μmol $P_i \cdot min^{-1} \cdot mg^{-1}$ with incubation times not longer than 3 min, 345 000 mol. wt and was not stimulated by trypsin. By gel electrophoresis at alkaline pH (8.5) in 8 M urea or in sodium dodecylsulfate, the ATPase revealed a complex pattern with two major subunits (α and β) and two minor ones (γ and δ). The non-identity between the major subunits was demonstrated.

INTRODUCTION

Micrococcus lysodeikticus membrane ATPase can be released into solution by washing divalent cation-depleted membranes with low-ionic strength Tris · HCl buffers ("shock wash" release) [1, 2]. The "shock wash" enzyme had been purified by Sephadex filtration in 30 mM Tris · HCl buffer at pH 7.5 [3] or at pH 9.0 in 1 mM EDTA/50 mM NH_4HCO_3 [4]. None of the purified ATPases showed stimulation by trypsin [3, 4], unlike the enzyme bound to divalent cation-depleted membranes and most of the crude soluble preparations [2–4]. The enzyme purified by gel chromatography revealed the presence of two major subunits of molecular weight around 50 000 and two minor ones of mol. wt 41 500 and 28 500 [4]. This pattern was similar to that of mitochondrial [5], chloroplast [6] and *Escherichia coli* ATPases [7], but differed from membrane ATPases of other Gram-positive bacteria with regard to the minor subunits [8, 9].

From these studies, we concluded that the stimulation by trypsin of *M. lysodeikticus* ATPase did not solely depend on its minor subunits [2, 4]. To see whether or not the molecular properties of *M. lysodeikticus* ATPase were dependent on the purification procedure and how they correlated with its stimulation by trypsin,

we attempted to develop new procedures of purification of the "shock wash" ATPase. This report describes a faster method for the preparation of higher amounts of pure protein using preparative gel electrophoresis. The protein purified by both procedures shows the same properties.

MATERIALS AND METHODS

The crude "shock wash" ATPase was obtained as described earlier [1, 3] and concentrated to 10 mg protein/ml by continuous ultrafiltration under N_2 at 1–1.5 kg/cm^2 using a XM-50 Diaflo membrane. The concentrated enzyme was centrifuged at 100 000 $\times g$ for 1 h at 0 °C in order to remove some heavy, vesicular components. After centrifugation no significant loss of enzyme activity was observed.

Preparative electrophoresis was carried out in a Poly-Prep 200 apparatus from Buchler Co. No stacking gels were used. Separating gels with 6 % (w/v) acrylamide and 0.157 % (w/v), *N,N'*-methylenebisacrylamide (both chemicals from Fluka, Buchs, Switzerland) were employed. The gels (volume 80 ml, height 6 cm) were polymerized in 0.38 M Tris · HCl (pH 8.7) with 0.1 % (w/v) ammonium persulfate and 0.08 % (v/v) *N,N,N',N'*-tetramethylethylenediamine for 1 h. Buffers were as follows: (a) lower chamber: 0.4 M Tris · HCl (pH 8.0); (b) upper chamber: 50 mM Tris/53 mM glycine (pH 8.8); and (c) elution buffer: 100 mM Tris · HCl (pH 8.0). The electrophoreses were run at a constant current of 50 mA with a potential difference of about 200 V. The buffer circulation flow at 0 °C was approx. 25 ml/h. The effluent was continuously monitored for ultraviolet-absorbing material with a double-beam Uvicord III (LKB, Bromma, Sweden) equipped with a 206-nm fillter.

The conditions for analytical electrophoresis under non-dissociating conditions or in sodium dodecylsulfate (BDH Chem. Ltd, Poole, England) were described elsewhere [4]. The analytical gel system in urea at basic pH was run as follows: 5 % acrylamide/0.131 % bisacrylamide gels (0.6 $\times$ 4 cm or 10 cm), containing 8 M urea (Merck, Darmstadt, Germany) and 1 mM dithiothreitol (Calbiochem, Lucerne, Switzerland), were polymerized with 0.05 % (w/v) ammonium persulfate and 0.08 % *N,N,N',N'*-tetramethylethylenediamine (v/v) for 30 min at room temperature in 0.38 M Tris · HCl buffer (pH 8.7). The upper electrode chamber contained 5 mM Tris/37.5 mM glycine (pH 8.3), 8 M urea and 1 mM dithiothreitol. The lower chamber contained the same buffer but no urea. Dithiothreitol did not modify the electrophoretic patterns but increased their resolution.

ATPase activity was assayed with ATP/Ca^{2+} (1/1) as previously described [1, 3]. However, incubations were stopped at 3 min, since we noticed in parallel studies (Nieto, M., Muñoz, E., Carreira, J. and Andreu, J. M., unpublished observations) that the reaction was not linear after this time. Unit is redefined as the amount of enzyme able to liberate 1 μmol P_i per min and specific activities are normalized according to this type of units. Where stated, trypsin was added to the reaction mixture at the concentrations indicated.

RESULTS

Purification of the ATPase

The elution profile of ATPase activity in preparative gel electrophoresis is shown in Fig. 1. Table I summarizes the yield, specific activity and trypsin-stimulated

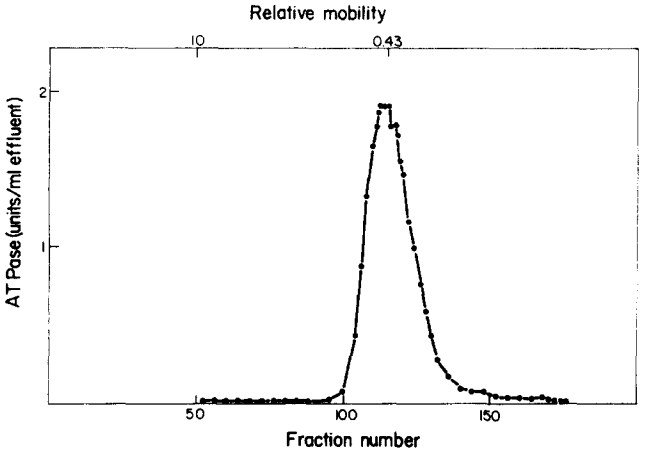

Fig. 1. Preparative gel electrophoresis of ATPase. Concentrated crude ATPase (about 100 mg protein) was pretreated with 1 mg ribonuclease (Koch Light Labs, Colnbrook, England) in presence of 0.5 mM EDTA for 20 min at room temperature and then for another 20 min, same temperature, with 1 mg deoxyribonuclease (Calbiochem) in 2 mM Mg^{2+} to provide an excess of Mg^{2+}. After addition of 20 % (w/v) saccharose and 100 μg bromophenol blue (Merck, Darmstadt, Germany) the enzyme was layered onto the gel. Each fraction was collected after 6 min of electrophoretic run and amounted to approx. 2.5 ml. Migration was from right to left (anode). The active fractions were pooled, concentrated by XM-50 ultrafiltration in the cold, and stored frozen (−20 °C) at 10 mg protein/ml in 50 mM Tris/H_2SO_4 (pH 7.5).

activity of the purified enzyme as compared with the crude soluble preparation. The yield is higher than those previously reported [3, 4]. The specific activity of the ATPase purified by gel electrophoresis (i.e. in 10 min incubation, when linearity was markedly reduced, it liberated 34 μmol P_i/mg protein) is comparable to those reported by us for Sephadex-purified preparations [3, 4] but lower than those reported by Salton and Schor [10, 11]. It is also worthwhile to mention the quickness of the procedure because six preparative runs allowed us to handle 100 g dry weight *M. lysodeikticus* cells in 2 weeks for obtaining the results recorded in Table I.

TABLE I

CHARACTERISTICS OF CRUDE AND PURIFIED ATPase PREPARATIONS FROM *MICROCOCCUS LYSODEIKTICUS*

ATPase preparations are described in the text and Fig. 1. For details of ATPase assay and definition of units see the text. Protein was measured as described elsewhere [3].

	Protein (mg)	ATPase activity (units)	Specific activity (units/mg protein)	Trypsin-stimulated activity (% control)
Crude enzyme	620	508	0.82	130
Enzyme purified by gel electrophoresis	55	385	7.0	80*, 100**

* Assayed with 0.2–0.5 mg trypsin/ml.
** Assayed with 4–30 μg trypsin/ml.

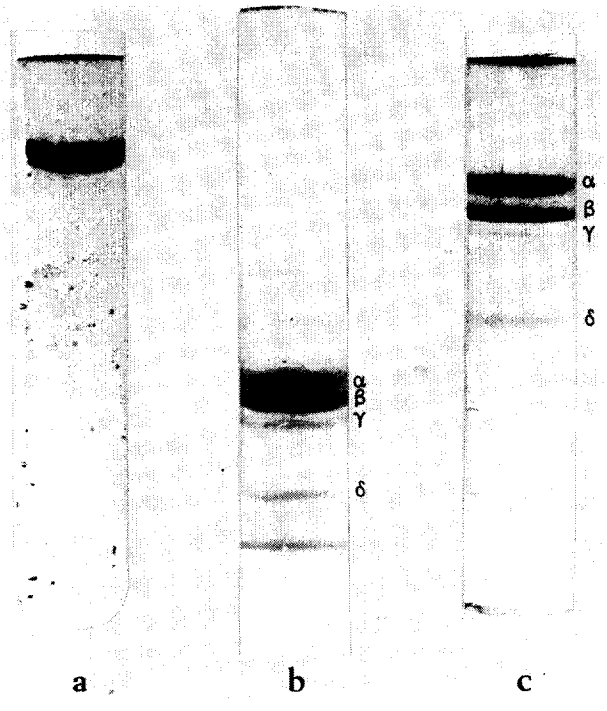

Fig. 2. Analytical gel electrophoresis of purified ATPase. (a) Non-dissociating conditions (40–50 μg protein). (b) Subunit pattern in sodium dodecylsulfate; samples (50–60 μg protein) were dissociated as described previously [4]. (c) Subunit pattern in the urea-basic pH system (see the text); samples (50–60 μg protein) were dissociated with 10 M urea for 30 min at 25 °C.

In agreement with previous findings, the ATPase purified by gel electrophoresis was not stimulated by trypsin and even inactivated when high concentrations of the protease were used. Its purity was checked by analytical gel electrophoresis (see Gel a in Fig. 2) and by sedimentation velocity experiments. A weight-average molecular weight of 345 000 was determined from sedimentation equilibrium of the pure protein at 0.5 mg protein/ml in 50 mM Tris · H_2SO_4 buffer (pH 7.5).

Subunit structure

The subunit structure of *M. lysodeikticús* ATPase purified by gel electrophoresis as revealed by its dissociation with dodecylsulfate and urea at pH 8.5 is illustrated in Fig. 2 (Gels b and c). Note the similarities between the two patterns with two predominant subunits (corresponding to the former α and β) and two minor ones (γ and δ). The fast-moving band also observed in these gels showed variable intensity depending on the preparation and electrophoretic run. As previously, it disappeared in 10 % acrylamide runs and very likely represented a mixture of small contaminants [4]. Thus, it was not considered as a subunit. Ferguson plots [12] of the major subunits in both dissociating systems confirm (Fig. 3) the non-identity between them, α having a slightly higher molecular weight than β in both systems. On the other hand, α must have less free negative charges than β in urea at alkaline pH. The separation

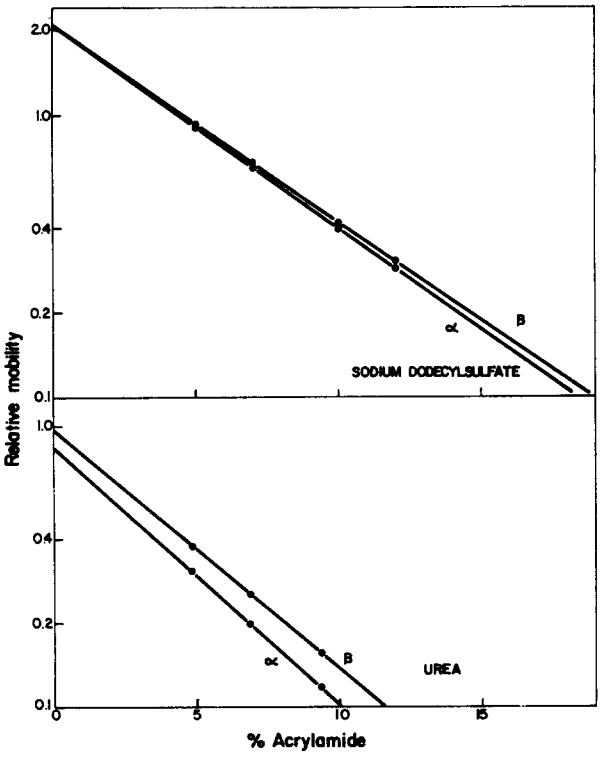

Fig. 3. Ferguson plots of the two major subunits of ATPase in sodium dodecylsulfate and in urea-basic pH.

of the two subunits in the urea system was the best at low concentrations of acrylamide.

DISCUSSION

Comparative studies with different types of mitochondrial ATPase, although somewhat in conflict, had been very helpful in elucidating the structural and functional roles of the protein [13]. We are now attempting a similar approach hoping to gain as much information for bacterial membrane ATPase as for mitochondrial ATPase, but perhaps less controversial. The ATPase purification to at least 95 % purity by gel electrophoresis represents an advantage in yield and speed when compared to former purification procedures and should allow further studies of the protein at the molecular level. Moreover, the preparation purified by preparative gel electrophoresis enabled us to confirm the quaternary structure of the ATPase, showing a relatively high proportion of γ, a subunit found previously to be weakly bound to the ATPase [4]. The molecular weight of 345 000 is similar to that previously reported [4] and probably reflects a complex without this subunit as previously suggested [4].

The analytical results obtained with the urea system indicate that the procedure must be potentially useful for the separation of the two major subunits.

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REFERENCES

1 Muñoz, E., Nachbar, M. S., Schor, M. T. and Salton, M. R. J. (1968) Biochem. Biophys. Res. Commun. 32, 539–546
2 Lastras, M. and Muñoz, E. (1974) J. Bacteriol. 119, 593–601
3 Muñoz, E., Salton, M. R. J., Ng, M. H. and Schor, M. T. (1969) Eur. J. Biochem. 7, 490–501
4 Andreu, J. M., Albendea, J. A. and Muñoz, E. (1973) Eur. J. Biochem. 37, 505–515
5 Brooks, J. C. and Senior, A. E. (1972) Biochemistry 11, 4675–4678
6 Nelson, N. Nelson, H. and Racker, E. (1972) J. Biol. Chem. 247, 7657–7662
7 Bragg, P. D., Davies, P. L. and Hou, C. (1973) Arch. Biochem. Biophys. 159, 664–670
8 Schnebli, H. P., Vatter, A. E. and Abrams, A. (1970) J. Biol. Chem. 245, 1122–1127
9 Mirsky, R. and Barlow, V. (1973) Biochim. Biophys. Acta 291, 480–488
10 Salton, M. R. J. and Schor, M. T. (1972) Biochem. Biophys. Res. Commun. 49, 350–357
11 Salton, M. R. J. and Schor, M. T. (1974) Biochim. Biophys. Acta 345, 74–82
12 Chrambach, A. and Rodbard, D. (1971) Science 172, 440–451
13 Senior, A. E. (1973) Biochim. Biophys. Acta 301, 249–277