Biochimica et Biophysica Acta, 436 (1976) 183–189

BBA 77314

MICROCOCCUS LYSODEIKTICUS MEMBRANE ATPase. EFFECT OF TRYPSIN ON STIMULATION OF A PURIFIED FORM OF THE ENZYME AND IDENTIFICATION OF ITS NATURAL INHIBITOR

J. CARREIRA, E. MUÑOZ, J. M. ANDREU and M. NIETO

Sección Bioquímica de Membranas, C.S.I.C., Velázquez, 144, Madrid-6 (Spain)

(Received December 5th, 1975)

SUMMARY

A soluble purified form of *Micrococcus lysodeikticus* ATPase (form B_{AT}, from strain B, active, trypsin-stimulated) was stimulated 100 % by trypsin and this stimulation was inhibited by preincubation of the protease with phenyl methyl sulphonylfluoride. This form of the enzyme was also stimulated 125–150 % by filtration on Sephadex G-200. Analysis by sodium dodecyl sulphate-gel electrophoresis showed that stimulation of this form of *M. lysodeikticus* ATPase was always accompanied by the disappearance of a subunit of mol. wt. 25 000 (ε subunit). It suggests that this subunit is the natural inhibitor of *M. lysodeikticus* ATPase. In the case of ATPase stimulation by trypsin, a partial and limited degradation of the α subunit was also observed. The interaction between the ε subunit and the rest of the ATPase complex was reversibly affected by pH, suggesting its non-covalent nature.

INTRODUCTION

The adenosine triphosphatase or ATPase of *Micrococcus lysodeikticus* resembles those of mitochondria and chloroplasts in its molecular properties and latency when bound to the membrane [1–5]. The latency might be related to the aerobic character of the microorganism and can be directed to prevent the hydrolysis of ATP produced by respiration [2, 4]. The latency was overcome by mild proteolysis of the membrane-bound ATPase [2] and this suggested the existence of an inhibitor of this bacterial ATPase similar to that of mitochondrial [6] and chloroplast [7] ATPases. Crude "shock-wash" soluble preparations of *M. lysodeikticus* ATPase assayed with 8 mM ATP/Ca^{2+} (1 : 1) showed a 50–100 % stimulation of their activity depending upon the ratio of protease to enzyme used [1, 2, 8, 9] but the purified ATPase from the original strain NCTC 2665 was only slightly stimulated by trypsin, i.e., 24 % [10] or not stimulated at all [1, 8]. Andreu et al. [1, 8] were thus unable to find a direct correlation between the presence of defined γ and δ subunits and a component(s) of relative mobility 1.0 and the enzyme stimulation by trypsin. However, some workers [10, 11] assumed that the stimulation by trypsin was due to the presence of three

undefined minor subunits, the major and more stable of which was probably related to the δ subunit of Andreu et al. [1, 8].

We have reported recently [9] the isolation from a sub-strain of *M. lysodeikticus* of a soluble, highly purified form of the ATPase, that could be stimulated by trypsin to 100 % of its basal activity. This ATPase preparation was a suitable system to study the molecular mechanisms of trypsin stimulation. When compared with the unstimulated forms of the enzyme, this form referred to hereafter as B_{AT} (from strain B, active, trypsin-stimulated) had an additional subunit (ε) and a multiplicity of α subunit(s) differing in size and/or charge [9].

This paper presents indirect but conclusive experiments on the identification of the ε subunit as the natural inhibitor of *M. lysodeikticus* ATPase. The interaction of the ε subunit with the rest of the ATPase complex appears to be disrupted by Sephadex filtration, as occurred with the specific inhibitor of the mitochondrial ATPase [12] and by pH. The analysis of the molecular transformations induced by trypsin during ATPase stimulation confirmed the involvement of the ε subunit in the inhibition of the ATPase and suggested a possible transformation of the α subunit as a requirement for maximal activity. These results, while extending the homologies between this bacterial ATPase and those of energy-transducing organelles, also point to a very subtle control of the hydrolytic activity of this bacterial ATPase.

MATERIALS AND METHODS

The growth characteristics and membrane preparation of *M. lysodeikticus* PNB (strain B) have been described elsewhere [9]. Preparations of form B_{AT} of *M. lysodeikticus* ATPase were obtained by preparative gel electrophoresis [9]. They were stimulated by trypsin up to 100 % and had basal specific activities at 37 °C (8 mM ATP/Ca^{2+}, pH 7.5) ranging from 2 to 5 μmol $P_i \cdot min^{-1} \cdot mg\ protein^{-1}$. The standard assay of ATPase activity was carried out as previously described [1, 2, 9]. Where stated, trypsin (Calbiochem) was used at the concentrations indicated in the legends of the figures. The stock solution in ethanol of the protease inhibitor, phenyl methyl sulphonylfluoride (Serva Feinbiochemica), was 20 mM. Aliquots of this solution were added to the aqueous incubation mixtures to give a final concentration of 0.4 mM in all cases. Gel electrophoresis (Tris glycine, pH 8.5$\pm$0.2) was carried out in refrigerated (4 °C) gels (12$\times$0.6 cm) as already reported [1]. Protein was estimated by the method of Lowry et al. [13] or from the $E_{1\,cm}^{1\%} = 6.96$ at 276 nm determined previously [14].

RESULTS AND DISCUSSION

The optimal molar ratio of trypsin to ATPase that gave maximum stimulation (100 %) in 5 min at 37 °C was 3/1 (i.e., a 1/5 ratio on a weight basis). The protease treatment was performed in the presence of 8 mM ATP/Ca^{2+} to protect the ATPase from extensive proteolysis. If the trypsin solution was preincubated with phenyl methyl sulphonylfluoride, the inhibited protease was no longer able to stimulate the ATPase activity (Fig. 1). On the other hand, the reactivated protease, obtained after spontaneous hydrolysis of its complex with the inhibitor (half-life 90 min), was again able

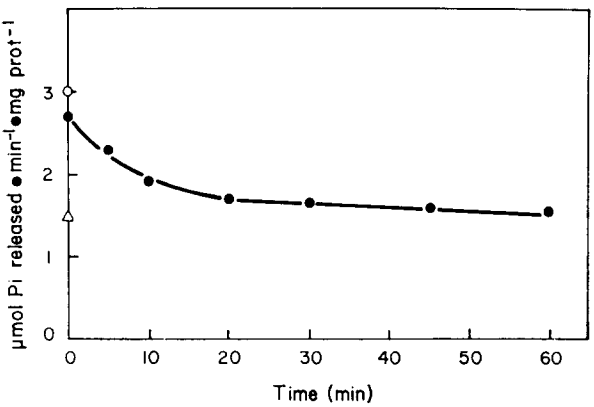

Fig. 1. Inhibition of trypsin-stimulation of *M. lysodeikticus* ATPase by preincubation of trypsin with phenyl methyl sulphonylfluoride. Trypsin (15 µg in 200 µl of 30 mM Tris/HCl, pH 7.5) was incubated with 0.4 mM phenyl methyl sulphonylfluoride for different times as indicated on the abscissa axis and then an aliquot of the mixture (3 µg protease/15 µg ATPase) was added to the standard assay of ATPase activity (●—●); △—△ represents the original basal ATPase activity and ○—○ the level of ATPase activity induced by non-inhibited trypsin. For details of the ATPase assay see the text and refs. 1, 2 and 9.

to stimulate the ATPase activity. Therefore, trypsin stimulation resulted from a proteolytic effect.

Analysis by sodium dodecyl sulphate-gel electrophoresis of form B_{AT} stimulated by trypsin under the optimum conditions deduced from Fig. 1, i.e. incubated with 3-fold molar excess of trypsin in the presence of 8 mM ATP/Ca^{2+}, did not allow any clear conclusion to be drawn. It showed a great amount of low molecular weight material together with a marked destruction of α and ε subunits. This indicated that the concentration of trypsin required to stimulate rapidly the ATPase was high and its proteolytic effect was seemingly too extensive during the experiment. Clearer results were obtained when the stimulation of form B_{AT} of *M. lysodeikticus* ATPase was induced by preincubating the enzyme in the absence of substrate with a lower trypsin concentration (trypsin/ATPase = 1/500, w/w). Under these conditions, maximum ATPase activity was obtained after 45 min incubation with the protease. This treatment destroyed almost completely subunit ε (mol. wt. 25 000) but left subunit δ unchanged (Fig. 2). It is worth noting that the α subunit was also degraded to give a main band that overlapped with subunit β and a new band of relative mobility 0.85. This transformation of the α subunit was perhaps necessary for maximum ATPase activity and must be different from that previously observed following inactivation of the enzyme [9, 14]. Other analytical methods besides dodecyl sulphate-gel electrophoresis would be required to characterize the different types of subunit α.

We knew from previous work [1, 2] that ATPase purification by Sephadex filtration yielded always unstimulated forms of *M. lysodeikticus* ATPase. Therefore, form B_{AT} (9 mg) purified by preparative gel electrophoresis was filtered through a column of Sephadex G 200 (V_o = 150 ml; V_i = 445 ml) packed and eluted with 30 mM Tris/HCl (pH 8.0). We obtained a non-stimulated form B_A, whose specific activity and dodecyl sulphate-subunit pattern are shown in Fig. 3 together with those

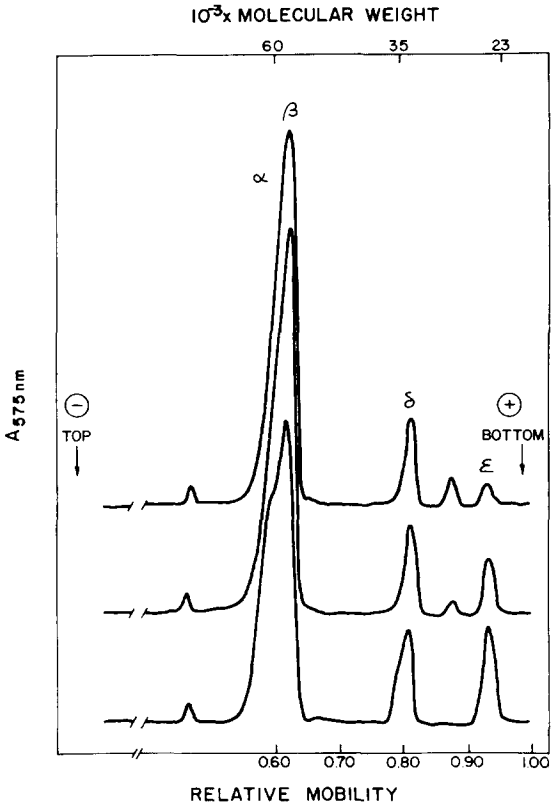

Fig. 2. Scans of sodium dodecyl sulphate electrophoretic patterns of form B_{AT} of *M. lysodeikticus* ATPase following its stimulation by trypsin as a function of time. Samples (100 μl of 30 mM Tris/HCl, pH 7.5) containing 35 μg ATPase were incubated with 0.07 μg trypsin at 37 °C for different times. Aliquots (6 μg) were used for measuring ATPase activity and the rest of the samples subjected to dodecyl sulphate-gel electrophoresis. Lower scan, 0 min incubation with trypsin; middle, after 30 min treatment with trypsin; upper, after 45 min treatment. The last sample had the maximum ATPase activity. Electrophoresis was carried out over the whole length of the gels to facilitate the separation of the low molecular weight components.

of the original form B_{AT} (a). As can be seen, the ε subunit was the common subunit lacking in the forms non-stimulated by trypsin. The components of relative mobility 1.0 were absent in the fresh preparation and appeared in the older preparation without noticeable change in the catalytic properties of the enzyme. Thus, these low molecular weight components did not arise from subunit ε, i.e., as a result of its degradation, and did not bear any relationship to the latency of the enzyme.

We also were aware from previous work that alkaline pH may induce the loss of trypsin stimulation [9]. When a form B_{AT} (spec. act. (pH 8.0) 6 μmol $P_i \cdot min^{-1} \cdot$ mg protein^{-1}; stimulated by trypsin to 10 μmol $P_i \cdot min^{-1} \cdot mg^{-1}$) stored in 30 mM Tris/HCl pH 8.0 was exposed for 2 h to 30 mM Tris/HCl pH 9.1, it lost completely the trypsin stimulation and increased the activity up to 15 μmol $P_i \cdot min^{-1} \cdot mg^{-1}$. This increase was higher than usual (see above and Fig. 3) because of the effect of alkaline pH [14]. If the pH was re-adjusted to a value close to 8, a 50–60 % decrease in

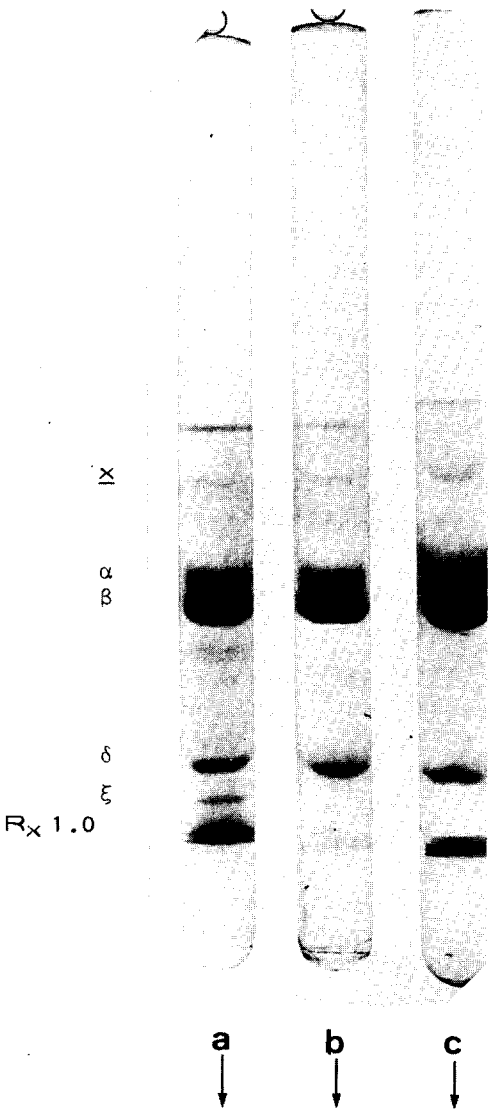

Fig. 3. Sodium dodecyl sulphate-electrophoretic patterns of trypsin-stimulated form B_{AT} and non-stimulated form B_A of *M. lysodeikticus* ATPase and comparison of their specific activities. Form B_{AT} was obtained by preparative gel electrophoresis [9], and form B_A by gel filtration of form B_{AT} (see the text). ATPase activity was measured using the standard assay [1, 2, 9] with 8 mM ATP/Ca^{2+} as substrate, 6–10 μg of purified enzyme with or without trypsin (1.5–2 μg) after 3 min incubation at 37 °C. (a) form B_{AT}; (b) form B_A freshly isolated; (c) form B_A after 1 week at 0 °C. Samples for electrophoresis contained 35–40 μg protein. ATPase activities were as follows (μmol · min^{-1} · mg $protein^{-1}$):

Fraction:	a	b	c
+Trypsin	10.0	13.5	13.3
−Trypsin	5.0	13.6	13.4

ATPase activity and a 25 % stimulation by trypsin were again observed. In other experiments the enzyme was only exposed to pH 9.1 for 5–10 min. In this case on returning to pH 8.0 the initial stimulation was fully recovered. Similar results were obtained by increasing the ionic strength (to about 0.3 — 0.4 M) at pH 8.0.

The easiest interpretation for this reversible loss of stimulation would be to assume that protein and inhibitor are involved in an association-dissociation equilibrium, the position of which is determined to a great extent by polar interactions. These interactions should be sharply affected by changes in the pH or the ionic strength of the medium. A weak, noncovalent interaction between inhibitor and protein was also suggested by the facility of their separation by gel filtration (see above). However, reassociation could not be attempted in this case because the dissociated inhibitor adhered very strongly to the Sephadex column and could not be eluted. Purification of the inhibitor by methods similar to those used in the case of mitochondrial ATPase [6] also failed. The difficulty probably arose, as in the case of chloroplast ATPase [7], from the hydrophobic character of the inhibitor.

All this indirect but conclusive evidence supports for the first time the existence of a natural inhibitor in *M. lysodeikticus* ATPase. This inhibitor seems to be a subunit of mol. wt about 25 000 (ε) which clearly differs from the γ and δ subunits and low molecular weight components as defined by Andreu et al. [1, 8]. It also proves that the assumption that several minor subunits were involved in the latency of *M. lysodeikticus* ATPase [10, 11] was not totally correct. Previous work from Nieuwenhuis et al. [15] and from our laboratory [16] also suggested the existence of a natural inhibitor of *Escherichia coli* ATPase. This would strengthen the similarities between organelle and bacterial energy-transducing ATPases. It would therefore be reasonable to refer to the soluble, purified bacterial ATPases as BF_1 factors [5].

ACKNOWLEDGEMENTS

J.C. thanks the Plan de Formación del Personal Investigador for a fellowship. The work was supported by a grant from Fondo Nacional para el Desarrollo de la Investigación. We are indebted to Miss Pilar Palacios for skillful technical assistance.

REFERENCES

1 Andreu, J. M., Albendea, J. A. and Muñoz, E. (1973) Eur. J. Biochem. 37, 505–515
2 Muñoz, E., Salton, M. R. J., Ng, M. H. and Schor, M. T. (1969) Eur. J. Biochem. 7, 490–501
3 Penefsky, H. S. (1974) in The Enzymes (Boyer, P. D., ed.),Vol. X, pp. 375–394, Academic Press, New York
4 Tikhonova, G. V. (1974) in Biochem. Soc. Special Publication No. 4 (Bronk, J. R., ed.), pp. 131–143, The Biochemical Society, London
5 West, I. C. (1974) in Biochem. Soc. Special Publication No. 4 (Bronk, J. R., ed.), pp. 27–38, The Biochemical Society, London
6 Pullman, M. E. and Monroy, G. C. (1963) J. Biol. Chem. 238, 3762–3769
7 Nelson, N., Nelson, H. and Racker, E. (1972) J. Biol. Chem. 247, 7657–7662
8 Andreu, J. M. and Muñoz, E. (1975) Biochim. Biophys. Acta 387, 228–233
9 Carreira, J., Andreu, J. M., Nieto, M. and Muñoz, E. (1976) Mol. Cell. Biochem., 10, 67–76
10 Salton, M. R. J. and Schor, M. T. (1974) Biochim. Biophys. Acta 345, 74–82
11 Salton, M. R. J. and Schor, M. T. (1972) Biochem. Biophys. Res. Commun. 49, 350–357
12 Racker, E. and Horstman, L. L. (1967) J. Biol. Chem. 242, 2547–2551

13 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
14 Nieto, M., Muñoz, E., Carreira, J. and Andreu, J. M. (1975) Biochim. Biophys. Acta 413, 394–414
15 Nieuwenhuis, F. J. R. M., Drift, J. A. M. v. d., Vect, A. B. and Van Dam, K. (1974) Biochim. Biophys. Acta 368, 461–463
16 Carreira, J., Leal, J. A., Rojas, M. and Muñoz, E. (1973) Biochim. Biophys. Acta 307, 541–556