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ISOLATION AND CHARACTERIZATION OF AN INHIBITORY SUBUNIT OF THE $Mg^{2+} - Ca^{2+}$-ATPase OF *ESCHERICHIA COLI*

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SUMMARY

1. Stimulation of the *Escherichia coli* ATPase activity by urea and trypsin shows that the ATPase activity both in the membrane-bound and the solubilized form is partly masked.

2. A protein, inhibiting the ATPase activity of *Escherichia coli*, can be isolated by sodium dodecyl sulphate polyacrylamide gel electrophoresis of purified ATPase. The inhibitor was identified with the smallest of the subunits of *E. coli* ATPase.

3. The molecular weight of the ATPase inhibitor is about 10 000, as determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis and deduced from the amino acid composition.

4. The inhibitory action is independent of pH, ionic strength or the presence of Mg^{2+} or ATP.

5. The ATPase inhibitor is heat-stable, insensitive to urea but very sensitive to trypsin degradation.

6. The *Escherichia coli* ATPase inhibitor does not inhibit the mitochondrial or the chloroplast ATPase.

INTRODUCTION

The energy-transducing ATPase present in the inner membrane of *Escherichia coli* is a large multimeric protein, the subunit composition of which resembles that of ATPases from mitochondria and chloroplasts [1, 2]. One of the most striking similarities between the bacterial, chloroplast and mitochondrial ATPase is the stimulation by trypsin of the ATPase activity [3–6]. In mitochondria [7–9] and chloroplasts [10], the presence of a naturally occurring inhibitor has been demonstrated, which is responsi-

Abbreviations: ATPase, adenosine triphosphatase [EC 3.6.1.3]; ACMA, 9-amino-6-chloro-2-methoxyacridine; S_{13}, 5-chloro-3-*t*-butyl-2′-chloro-4′-nitrosalicylanilide; TES, *N*-tris(hydroxymethyl) methyl-2-aminoethane sulphonic acid; SDS, sodium dodecyl sulphate; DCCD, *N,N′*-dicyclohexylcarbodiimide; TosPheCH_2Cl-trypsin, trypsin treated with L-1-tosylamido-2-phenylethylchloromethyl ketone, a specific chymotrypsin inhibitor.

ble for the masking of the ATPase activity. In both cases the inhibitor is a low-molecular weight, trypsin-sensitive protein and (probably) part of the ATPase molecule itself. It inhibits both the membrane-bound and the solubilized ATPase. The inhibition in mitochondria is dependent upon pH, salt concentration, Mg^{2+} and ATP [8, 9, 11, 12].

Furthermore, Ernster et al. [13, 14] and van de Stadt et al. [12] have presented strong evidence that this mitochondrial inhibitor plays a regulatory role in oxidative phosphorylation. It controls the backflow of energy from ATP to energy-linked processes but has little effect on ATP synthesis. This regulatory function has also been postulated for chloroplasts [10, 15]. If such an inhibitor is an essential component of the system catalysing oxidative phosphorylation, it may be expected that it is also present in bacteria.

The first indication that bacteria also contain an endogenous ATPase inhibitor came from experiments showing stimulation by trypsin of the ATPase activity in several types of bacteria, such as *Micrococcus lysodeikticus* [5], *Azotobacter vinelandii* [6], *E. coli* [16, 17] and *Salmonella typhimurium* [18]. There are, however, also bacteria, for example the obligate-anaerobe *Streptococcus faecalis*, which possess an ATPase activity which cannot be stimulated by trypsin. As pointed out by West [19], this bacterium probably uses its ATPase only hydrolytically to drive transport of solutes at the expense of ATP. Although it does not seem necessary for each bacterium to possess a natural ATPase inhibitor, we shall present evidence in this paper that at least one bacterium, *E. coli*, possesses a natural ATPase inhibitor.

METHODS

E. coli strain A 428 was used. Sub-bacterial particles (P^+) and crude coupling factor were isolated as described previously [20].

E. coli (bacterial) ATPase was isolated essentially according to the procedure of Nelson et al. [21]. Beef-heart mitochondrial ATPase and beef-heart mitochondrial-ATPase inhibitor were isolated according to the procedures described in refs. 22 and 12, respectively. Chloroplast ATPase was prepared as described by Strotmann et al. [23]. Where chloroplast ATPase inhibitor was used, chloroplast ATPase (1 mg/ml) was treated with 7 M urea according to Nelson et al. [10].

ATPase activity was assayed by measurement of inorganic phosphate [24] liberated from ATP at 37 °C. The incubation mixture, unless stated otherwise, routinely contained 50 mM Tris · HCl, 5 mM ATP, 5 mM phosphoenolpyruvate, 2.5 mM $MgCl_2$ and 30 μg pyruvate kinase (final volume 1 ml, pH 8.0).

Protein was determined by the method of Lowry et al. [25] with bovine serum albumin as standard.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (disk or slab gels) was carried out as described by Laemmli [26] and Ames [27].

Tos$PheCH_2Cl$-trypsin and soy-bean trypsin inhibitor were obtained from Worthington.

RESULTS

In an earlier report [17], we reported evidence for the presence of a naturally

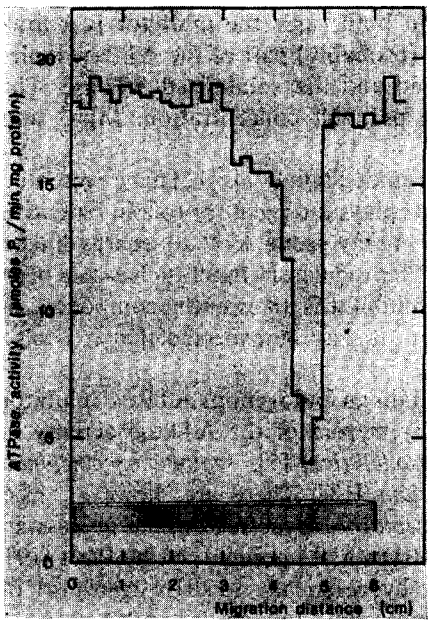

Fig. 1. Inhibition of the ATPase activity of *E. coli* ATPase by gel-electrophoresis fractions of bacterial ATPase. Electrophoresis of bacterial ATPase on 12 % sodium dodecyl sulphate polyacrylamide gels was done in duplicate. One gel (5 μg bacterial ATPase) was stained with Coomassie blue (inset) and the other (100 μg bacterial ATPase) was cut in 2 mm pieces which were immersed into 0.25 ml of a medium containing 2 mM TES (pH 6.5), 5 mM dithiothreitol and 8 mg/ml bovine serum albumin. Slices were soaked overnight at 4 °C and the ATPase-inhibitor activity of 50 μl was tested on the ATPase activity of bacterial ATPase. For this, 0.5 μg bacterial ATPase was preincubated for 20 min at 37 °C in 0.5 ml 2 mM TES, 1 mM $MgCl_2$, 1 mM ATP, pH 6.3, with 50 μl of each fraction and ATPase activity was measured as described in Methods.

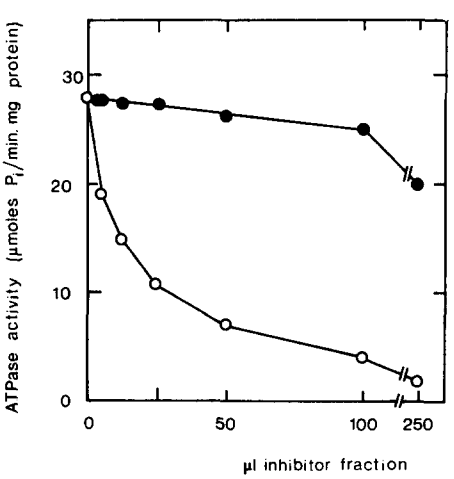

Fig. 2. Titration of the effect of the inhibitor on the ATPase activity of *E. coli* ATPase. Bacterial ATPase (100 μg) was electrophoresed on a 12 % SDS polyacrylamide gel. The inhibitory part of the gel (containing only the ε-subunit) was soaked overnight in 2.5 ml medium as described in Fig. 1. Different amounts of this fraction were tested on bacterial ATPase (0.5 μg) as described in Fig. 1. ATPase activity was measured as described in Methods. ●—●, control part of the gel, also soaked in the same medium; ○—○, inhibitory fraction.

TABLE I

INFLUENCE OF Mg^{2+} AND ATP ON THE ASSOCIATION OF THE *E. COLI* ATPase AND ITS INHIBITOR

E. coli ATPase (0.5 μg) was preincubated for 20 min at 37 °C in different buffers containing 2 mM TES (pH 6.5), 1 mM $MgCl_2$, 1 mM ATP in the presence of 50 μl inhibitor fraction (plus inhibitor) or a control addition (minus inhibitor) (see Fig. 2). ATPase activity was measured as described in Methods.

Buffer	μmol P_i/min · mg protein		% Inhibition
	Minus inhibitor	Plus inhibitor	
TES	65.4	28.0	57
TES+ATP	48.1	22.3	54
TES+Mg^{2+}	52.7	21.5	59
TES+Mg^{2+}+ATP	47.9	20.5	57

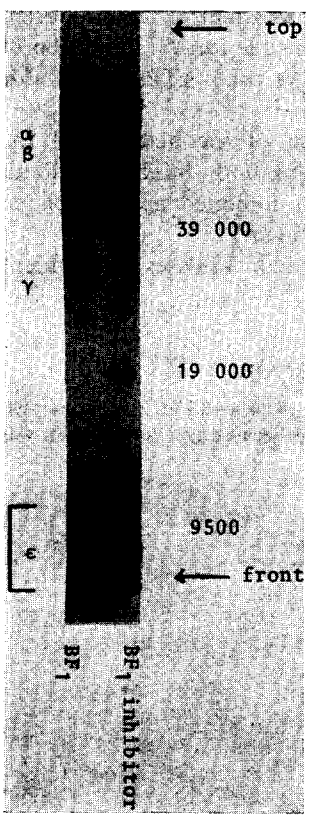

Fig. 3. Gel electrophoresis of *E. coli* ATPase and its inhibitor. 1 mg bacterial ATPase was electrophoresed on 12 % preparative SDS polyacrylamide gels. Part of the gel, containing only the ε-subunit (as indicated in the figure) was immersed in 10 ml medium containing 5 mM TES (pH 6.5), 5 mM dithiothreitol and soaked overnight at 4 °C. These 10 ml were dialysed against water for 4 days with several changes of water. The resulting dialysate was freeze-dried. Part of it was taken up in 2 % SDS, 2 % β-mercaptoethanol and Tris buffer (pH 6.5) and boiled at 100 °C for 5 min. It was then co-electrophoresed with 10 μg bacterial ATPase on a 12 % SDS-polyacrylamide slab gel according to Ames [27]. BF_1: bacterial ATPase.

occurring ATPase inhibitor protein in *E. coli*. It was suggested that the 12 000 molecular weight protein was the inhibitor (ε-subunit of bacterial ATPase).

That the ε-subunit of the *E. coli* ATPase is indeed the ATPase inhibitor can be seen from Fig. 1. This figure illustrates the rapid isolation of the ATPase inhibitor by SDS-polyacrylamide gel electrophoresis of bacterial ATPase according to the method described by Satre et al. [8]. In our case, about 100 μg *E. coli* ATPase were electrophoresed on a 12 % SDS-polyacrylamide gel, which was subsequently cut into pieces of 2 mm. These pieces were soaked overnight in 2 mM TES (pH 6.5), 5 mM dithiothreitol and 8 mg/ml bovine serum albumin (to bind the SDS present in the gel slice). When we tested the inhibitory activity of these fractions, it became clear that the inhibitory activity coincided with the position of the ε-subunit of the bacterial ATPase. If we electrophoresed a trypsin-treated bacterial ATPase preparation, we found no protein at the position of the ε subunit nor any inhibitory activity in the gel. This proves that the inhibitor is irreversibly destroyed by the trypsin treatment (see also Table III). Very recently, Smith et al. [28] reported similar results; they also found that the ε-subunit is responsible for the inhibition of the ATPase activity. For all the following experiments we used ATPase inhibitor obtained by gel electrophoresis.

Fig. 2 shows a titration curve of the inhibition of the activity of the *E. coli* ATPase by the inhibitor. It can be seen that the inhibitor inhibits by about 80–90 %. Also, the membrane-bound ATPase of *E. coli* is inhibited by the isolated inhibitor in the same way as the solubilized ATPase (not shown).

The association between the different inhibitors and their ATPases is dependent

TABLE II

AMINO ACID COMPOSITION OF THE *E. COLI*-ATPase INHIBITOR

The freeze-dried dialysate containing the bacterial ATPase inhibitor (see legend Fig. 3) was analysed for amino acid composition. Glycine could not be estimated accurately because even long dialysis was not sufficient to remove all the electrophoresis buffer, Tris-glycine.

Amino acid	Mol %	(Mol. weight 10 000) Residues per mol
Asp	9.75	8
Thr	4.35	3
Ser	10.6	8
Glu	18.75	15
Pro	4.3	3
Gly	n.d.	n.d.
Ala	10.3	8
Lys	2.6	2
Val	7.05	6
Met	1.25	1
Ile	4.95	4
Leu	9.05	7
Tyr	0	0
Phe	3.4	3
Lys	5.95	5
His	3.0	2
Arg	4.9	4

upon several conditions. In the case of the beef-heart mitochondrial ATPase both ATP and Mg^{2+} are required [12, 11]; for the yeast mitochondrial ATPase, the presence of ATP alone is sufficient [8]. Also for a bacterium *M. lysodeikticus* [29], it is known that Mg^{2+} can influence the ATPase activity, possibly by changing the interaction between the ATPase and its inhibitor. Table I shows that in *E. coli* neither ATP nor Mg^{2+} are necessary for the association of the bacterial ATPase and its inhibitor. Also the chloroplast ATPase inhibitor [10] does not require Mg^{2+} or ATP.

Another similarity between the *E. coli* ATPase inhibitor and the chloroplast ATPase inhibitor is that their action is pH-independent (data not shown); this is in contrast to the mitochondrial inhibitor which only works at low pH [7, 8, 11].

A third similarity of the *E. coli* ATPase inhibitor and chloroplast ATPase inhibitor is the lack of an effect of salts, which were noted to interfere with the binding of the beef-heart mitochondrial ATPase inhibitor to the beef-heart mitochondrial ATPase [7].

The heat stability [7, 10] is together with the high trypsin sensitivity a common property of all natural ATPase inhibitors. The *E. coli* ATPase inhibitor shares this property. Treatment of the inhibitor during 8 min at 90 °C does not destroy its inhibitory activity.

Co-electrophoresis of the bacterial ATPase inhibitor with purified bacterial ATPase (Fig. 3) shows again that the ATPase-inhibitor protein may be identified with the smallest subunit (ε) of the bacterial ATPase molecule itself. The molecular weight of the inhibitor protein is about 9500, as determined by SDS-polyacrylamide gel electrophoresis. It is noteworthy that in some experiments, dimers and tetramers of

TABLE III

SPECIFICITY OF INHIBITION BY NATURAL INHIBITORS OF THE *E. COLI*, CHLOROPLAST AND BEEF HEART MITOCHONDRIAL ATPase ACTIVITY

0.4 μg bacterial ATPase was incubated as described in Fig. 1, with 100 μl bacterial ATPase inhibitor, 2 μg beef-heart mitochondrial ATPase inhibitor or 50 μl of a 7 M urea-treated chloroplast ATPase (1 mg/ml). ATPase activity was assayed as described in Methods. 15 μg chloroplast ATPase, heat-activated according to Nelson et al. [10], was incubated for 5 min in 0.5 ml 50 mM Tris · HCl (pH 8.0) with 50 μl bacterial ATPase inhibitor or 50 μl of a 7-M urea-treated chloroplast ATPase (1 mg/ml). Ca^{2+}-ATPase activity was assayed as described in Methods, with 10 mM $CaCl_2$ instead of 2.5 mM $MgCl_2$. 1 μg beef heart mitochondrial ATPase was incubated with 50 μl bacterial ATPase inhibitor or 2 μg beef heart mitochondrial ATPase inhibitor as described in Fig. 1. ATPase activity was measured as described in Methods. In the experiments with trypsin-treated bacterial ATPase inhibitor, 50 μl or 100 μl inhibitor was preincubated with 100 μg TosPheCH_2Cl-trypsin. TosPheCH_2Cl-trypsin was inhibited after 20 min with 200 μg trypsin inhibitor (Soy bean). The specific activities of bacterial, chloroplast and mitochondrial ATPase were respectively, 74, 22 and 64 μmol P_i/min · mg protein. The numbers in the figure give the percentage inhibition.

Inhibitors	ATPase		
	Beef-heart	Chloroplast	*E. coli*
Beef heart	52	n.d.	2
Chloroplast	n.d.	68	0
E. coli	25	0	72
E. coli (trypsin-treated)	23	0	7

the inhibitor were found; a similar association was reported earlier for the beef heart mitochondrial ATPase inhibitor [30].

The amino acid composition of the inhibitor is shown in Table II. Like the chloroplast ATPase inhibitor [10], it does not contain tyrosine. The polarity of the bacterial ATPase inhibitor also is about the same as that of the chloroplast ATPase inhibitor and differs from that of the beef-heart mitochondrial ATPase inhibitor [8] and the yeast mitochondrial ATPase inhibitor [8]. The minimal molecular weight as determined by the amino acid composition is about 10 500.

Up till now only the mitochondrial ATPase inhibitor from beef-heart and yeast were interchangeable [8, 31, 32]. The chloroplast ATPase inhibitor does not inhibit the mitochondrial ATPase and the mitochondrial ATPase inhibitor does not inhibit the chloroplast ATPase [10]. In this respect, we were not suprised to find in our experiments that the *E. coli* ATPase inhibitor does not inhibit the beef heart mitochondrial or the chloroplast ATPase (Table III). The 25 % inhibition of beef-heart mitochondrial ATPase by the *E. coli* ATPase inhibitor is probably due to the presence of SDS, not bound to the bovine serum albumin, since after destroying the inhibitor by trypsin this percentage of inhibition still remained.

DISCUSSION

The similarity between the mitochondrial, chloroplast and bacterial ATPases which play a major role in oxidative phosphorylation has been discussed in detail elsewhere [1, 2, 33]. The inhibitor protein is one of the less well-defined components of this ATPase, and has been observed in some organisms, but not in others. It is tempting to conclude from the results presented in this paper that the ATPase inhibitor is probably an essential part of all ATPases. The question remains: what is the role of this inhibitor in the different organisms and how does it act?

Concerning the last question, experiments with *E. coli* [28] and chloroplasts [34] indicate that when the γ-subunit of the ATPase is absent, the inhibitor cannot bind to the ATPase molecule. Also antibody-studies with chloroplasts [10] indicate that the γ-subunit at least is involved in the binding of the inhibitor.

In spite of some similarities between the different ATPase inhibitors, i.e. molecular weight, trypsin sensitivity and heat stability, there are also some essential differences, which make it difficult to propose a general mechanism of action. The sophisticated regulation of ATP hydrolysis by the ADP/ATP ratio and the energy state of the membrane in beef heart mitochondria as proposed by Van de Stadt et al. [12] is not likely to be present in other organelles such as chloroplasts or bacteria such as *E. coli*, because no influence of ATP on the percentage inhibition of the inhibitor could be found. A second difficulty is the varying inhibition of the ATPase by its inhibitor. Some organisms have a completely inhibited ATPase (for instance *A. vinelandii*) while others (for instance *E. coli*) have only a partially inhibited ATPase.

The inhibition by the inhibitor can be changed in mitochondria through coupled substrate oxidation [12] and in chloroplasts through light plus dithioerythritol [35]. Despite several attempts, a similar effect could not be established in bacteria like *E. coli* and *A. vinelandii* (Nieuwenhuis, F. J. R. M. and Bakkenist, A. R. J., unpublished) and *Paracoccus denitrificans* (P. John quoted in ref. 36). Interestingly, in all these cases the inhibitor functions as a one-way regulator of the membrane-bound ATPase : ATP hydrolysis is inhibited, yet ATP synthesis is not [5, 12, 13].

It has been proposed for mitochondria [12] and chloroplasts [10, 37], that the inhibitor can be bound to the ATPase in a non-inhibiting way. This proposal could explain the observation that a fully active *E. coli* ATPase (about 100 units/mg) still contains a considerable amount of inhibitor [28]. We propose that the equilibrium between the "non-inhibited state" and the "inhibited state" is different in different organisms. It is, however, not possible to predict whether the origin of these different equilibria depends on the state or the amount of the inhibitor or on the state of the active part of the ATPase. In this regard we can imagine that obligate aerobic bacteria contain a completely inhibiting inhibitor because they do not have to use their ATPase hydrolytically. They induce the energized state or drive their solute transport by respiration alone. The obligate or facultative anaerobes (i.e. *E. coli*), which sometimes have to use their ATPase to drive transport [38], have most of their inhibitor in the "non-inhibitory state" [19].

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