Biochimica et Biophysica Acta, 429 (1976) 1020—1028

BBA 67775

Me^{2+}-(13 S) ATPase FROM *MICROCOCCUS* SP. ATCC 398E *

THE EFFECT OF TRYPSIN ON THE PURIFIED ENZYME

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(Received July 31st, 1975)
(Revised manuscript received December 5th, 1975)

Summary

By trypsin treatment of highly purified ATPase (EC 3.6.1.3) from *Micrococcus* sp. ATCC 398E, two enzyme modifications have been obtained.

(i) ATPase T_a, which has about the same activity as untreated ATPase.

(ii) A protein complex T_i, which lacks ATPase activity, but nevertheless binds ATP as shown by affinity chromatography.

Trypsin primarily shortens the α-chains of the "native" enzyme to α'-chains and removes the γ-subunit, thus yielding ATPase T_a. The formation of the protein complex T_i appears to be due to additional cleavage of one α'-chain into at least two more fractions.

Introduction

Me^{2+}-ATPase (EC 3.6.1.3) from *Micrococcus* sp. ATCC 398E purified by several procedures [2,3] shows evident similarities to ATPases of mitochondria [4—8], chloroplasts [4,9,10] and other bacteria, e.g. *Micrococcus lysodeikticus* [11—16], *Escherichia coli* [17—21], *Salmonella typhimurium* [21], *Bacillus megaterium* [22,23], *Streptococcus faecalis* [24,25], *Bacillus stearothermophilus* [26], and *Rhodospirillum rubrum* [27] with respect to molecular weight, subunit structure, and amino acid composition. These ATPases are involved in functions such as oxidative phosphorylation [4,18], photophosphorylation [4,27], and/or active transport [18,24,28].

* Although having been suggested as the neotype strain of *Micrococcus luteus*, the strain ATCC 398E does not fit all criteria obligate to *M. luteus* [1].

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The effect of trypsin on ATPases, originally used to release latent ATPase activity [14,29,30], has recently been studied in more detail in the case of *E. coli* ATPase [19,21].

In the present paper, we describe the effect of trypsin on purified ATPase from *Micrococcus* sp. ATCC 398E regarding activity, affinity to ATP, subunit structure, and molecular weight. This ATPase can be obtained as an enzyme complex consisting only of α-, β- and γ-subunits [2]. Our results also lead to some conclusions with respect to the subunit stoichiometry of the enzyme.

Experimental

Materials

Acrylamide, recrystallized four times, was purchased from Roth, Karlsruhe. Trypsin (purissimum, free from chymotrypsin), chymotrypsin, *N,N'*-methylenebisacrylamide, *N,N,N',N'*-tetramethylethylenediamine (TMED), sodium dodecyl sulfate, Coomassie brilliant blue G 250, β-mercaptoethanol were obtained from Serva, Heidelberg. DNAase purity grade I and II, phosphorylase *a*, albumin from bovine serum, albumin from hen egg, γ-globulin, pepsin, cytochrome *c*, and myoglobin were from C.H. Boehringer, Mannheim. All other chemicals were of analytical grade, purchased from E. Merck, Darmstadt.

Preparation of highly purified ATPase

ATPase was prepared from *Micrococcus* sp. ATCC 398E as published elsewhere [2]. The method involves the preparation of a crude extract by osmotic shock treatment of protoplasts at low ionic strength. The ATPase was then purified to homogeneity by $(NH_4)_2SO_4$ precipitation, chromatography on BioGel A 1.5 m, and preparative disc electrophoresis (5% acrylamide). The purified enzyme showed a single sharp band in the analytical disc electrophoresis on 5% polyacrylamide gels.

Treatment of ATPase with trypsin

To the protein solution (50 mM Tris · HCl buffer, pH 7.5) an equal volume of trypsin solution (50 mM Tris · HCl buffer, pH 7.5) was added yielding molar trypsin/ATPase ratios of 1 : 15. The incubation was carried out in 1 ml solution at 25°C.

After trypsin treatment, the samples were immediately subjected to either analytical or preparative disc electrophoresis to remove trypsin and separate the modified proteins. Activity tests were run with aliquots of the trypsin-treated samples.

Preparative disc electrophoresis

Preparative disc electrophoresis was performed with a Shandon Southern apparatus. Separating gels (45 ml, 11 cm) were prepared in the same way as for the analytical disc electrophoresis (see below). No sample gels were used. Cathode, anode, and elution buffers were the same (1.5 g/l Tris, and 7.2 g/l glycine, pH 8.5). The electrode buffers were recycled during operation.

For the first 30 min the current was 20 mA at 250 V, then it was raised to 50 mA at 500 V. After 2.5 h the front appeared and after 5 h the ATPase was eluted. The gel temperature was kept at 20°C.

Analytical disc electrophoresis

5% polyacrylamide separating gels were polymerized in the presence of 0.12% *N,N'*-methylenebisacrylamide, 0.07% ammonium peroxodisulfate, 0.04% TMED, 0.375 M Tris buffer pH 9.0. Sample gels were not used. The electrophoresis buffer contained 0.6 g/l Tris · HCl and 2.88 g/l glycine (at pH 8.5). The samples were run at 200 V and 4 mA/gel with bromophenol blue as front marker. The protein bands were stained with Coomassie brilliant blue G 250.

Analytical sodium dodecyl sulfate disc electrophoresis

The procedure of Weber and Osborn [31] was applied using 7.5% acrylamide gels. The buffer (0.1 M sodium phosphate at pH 7.2) contained 0.1% sodium dodecyl sulfate. The protein samples were depolymerized in the presence of 2% sodium dodecyl sulfate and 1% β-mercaptoethanol at 37°C for 2 h and then at 100°C for 3 min in the above buffer. The electrophoretic separation was carried out at 40 V and 15 mA per tube for 7 h. The protein bands were stained with Coomassie brilliant blue G 250. The gels were scanned at 600 nm with a Gilford spectrophotometer model 2400 equipped with a linear transporter.

The molecular weights of the ATPase subunits were determined using phosphorylase *a*, albumin from bovine serum, albumin from hen egg, γ-globulin, pepsin, cytochrome *c*, and myoglobin as standards.

Affinity chromatography

Iminobispropylamine-*N*-acetylhomocysteyl spacers were bound to BrCN-activated Sepharose 4B according to Porath et al. [32] and Cuatrecasas [33].

The spacer was linked to the 6-carbon of purineriboside triphosphate via a thioether bond. The synthesis of the matrix with affinity for ATP and its application to purification of an $(Na^+ + K^+)$-ATPase from bovine brain and an Me^{2+}-ATPase from *Micrococcus* sp. ATCC 398E are described elsewhere [34,3]. 2 ml of trypsin treated ATPase (0.1 mg/ml) was analyzed on a column packed with 4 ml affinity matrix at a flow rate of 2 ml/h. Previously the column had been equilibrated with 50 mM Tris · HCl buffer pH 7.5.

Fractions of 1 ml were collected. After fraction No. 10 the flow rate was raised to 10 ml/h. At fraction No. 41, a linear gradient of 0—25 mM ATP was applied to elute the proteins bound to the column. All procedures were carried out at 4°C.

Enzyme assay

The ATPase activity was determined continuously in the presence of 1 mM ATP and 10 mM $CaCl_2$ at 37°C by measurement of the liberated orthophosphate as described elsewhere [2].

One unit (U) is defined as the number of μmol of inorganic phosphate liberated during 1 min at 37°C. The specific activity is given in units per mg protein.

Protein determination

Protein was determined by a modified method of Lowry et al. [35].

Results and Discussion

Trypsin treatment of the electrophoretically purified ATPase from *Micrococcus* sp. ATCC 398E (consisting only of α-, β- and γ-subunits) results in a decrease in ATPase activity with a half life-time of 3 h under the conditions described in Experimental (see also Fig. 1).

Analytical disc electrophoresis on 5% polyacrylamide gels was performed immediately following tryptic digestion to stop the reaction. The electrophoretic experiments show that the single band of untreated ("native") ATPase N is converted into at least two fractions T_a and T_i after 1 h of digestion. These fractions move faster than ATPase N (Fig. 2). More extended incubation with trypsin resulted in a decrease of the T_a band being proportional to the increase of the T_i band. For further characterization the enzyme modifications T_a and T_i were separated by preparative disc electrophoresis on 5% polyacrylamide gel. Although partial overlapping could not be avoided, the front parts of the T_a band and the rear parts of the T_i band contained the pure protein fractions (Fig. 3).

The slower moving T_a band shows a specific activity of 10 ± 2 units/mg which is about the specific activity of the untreated enzyme [2] whereas the faster moving T_i band has no ATPase activity.

To investigate the binding of purineriboside phosphates to the ATPase proteins after trypsin treatment, we subjected ATPase T_i and ATPase T_a (isolated by preparative disc electrophoresis) to affinity chromatography [3] on a column packed with a matrix with a site that had an affinity for ATP. This was obtained by linking purineriboside triphosphate via *N*-acetylhomocysteyl-iminobispropylamine spacers to BrCN-activated Sepharose 4B. The results are shown in Fig. 4.

All fractions (untreated ATPase N, ATPases T_a and T_i) were bound to the matrix and eluted at the same concentration of ATP, indicating that the (or at least one) binding site of T_i still functions although this fraction is catalytically inactive.

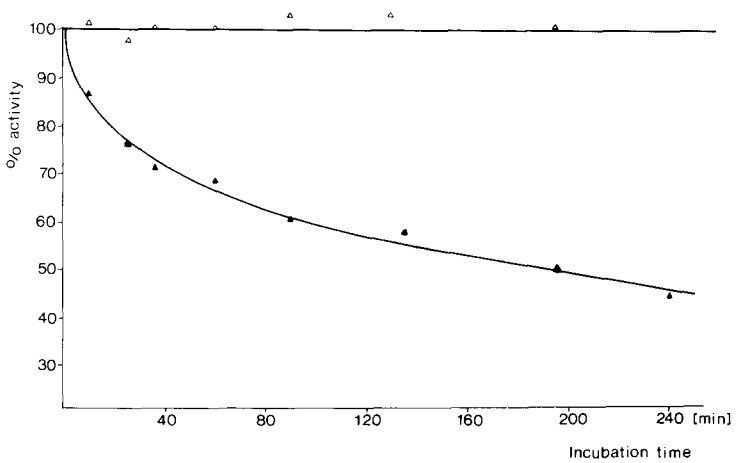

Fig. 1. Effect of trypsin on the activity of purified ATPase of *Micrococcus* sp. ATCC 398E (▲———▲). The treatment was carried out as described in Experimental. (△———△) control without protease.

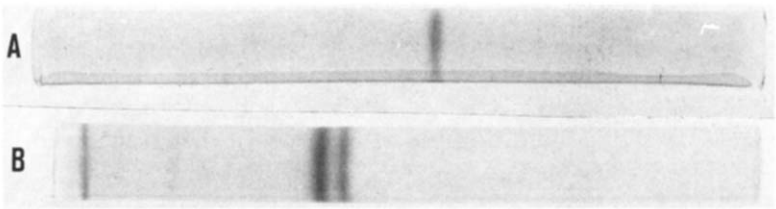

Fig. 2. Analytical disc electrophoresis on 5% polyacrylamide gels of (A) untreated ATPase (20 μg protein) and (B) enzyme treated for 3 h with trypsin, as described in Experimental (40 μg protein).

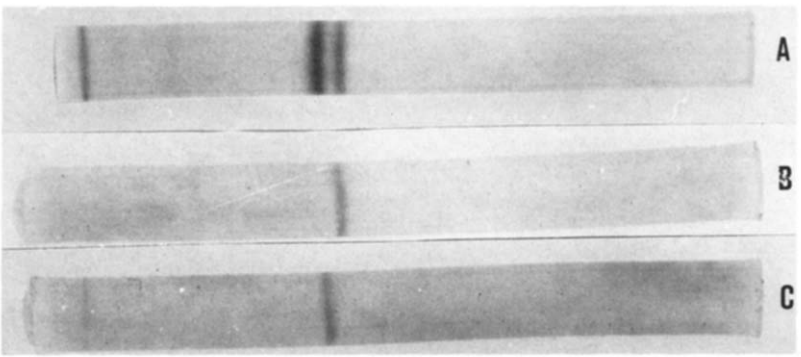

Fig. 3. Analytical disc electrophoresis on 5% polyacrylamide gels of ATPases T_a and T_i separated by preparative disc electrophoresis; (A) ATPases T_a and T_i obtained by 3 h treatment with trypsin (40 μg protein); (B) ATPase T_a (10 μg protein); (C) ATPase T_i (10 μg protein).

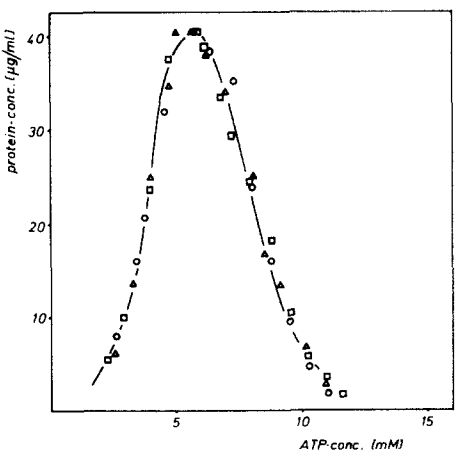

Fig. 4. Elution profile of ATPase N (●———●), ATPase T_a (■———■), and ATPase T_i (▲———▲) after binding to affinity matrix as described in Experimental. In each case 0.2 mg of protein was applied. Elution was followed by measuring protein concentration.

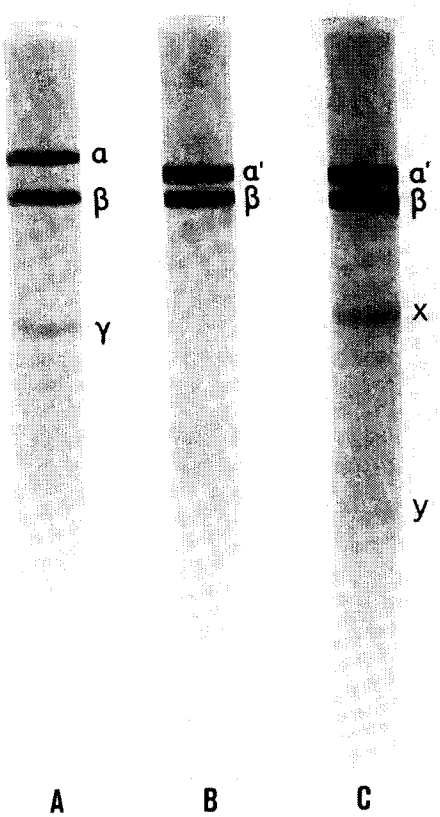

Fig. 5. Sodium dodecyl sulfate electrophoresis on 7.5% polyacrylamide gels of native and trypsin-modified ATPases. (A) untreated ATPase N (20 μg protein) M_R 65 000 (α), 55 000 (β), 35 000 (γ); (B) ATPase T_a (30 μg protein) M_R 61 000 (α'), 55 000 (β); (C) ATPase T_i (60 μg protein) M_R 61 000 (α'), 55 000 (β), 38 000 (x), 22 000 (y).

Fig. 5 shows the subunits of the three fractions (N, T_a, T_i) after treatment with 2% sodium dodecyl sulfate and 1% β-mercaptoethanol.

ATPase N (Me^{2+}-(13 S) ATPase from *Micrococcus* sp. ATCC 398E purified according to our procedure [2]) contains three different subunits with molecular weights of 65 000 (α), 55 000 (β), and 35 000 (γ).

ATPase T_a consists of slightly shortened α-chains (α'-chains) with a molecular weight of 61 000, apparently unchanged β-chains, but no γ-chains (see also Fig. 6a).

ATPase T_i consists of α'-chains, apparently unchanged β-chains, and two further protein fragments with molecular weights of 38 000 (x) and 22 000 (y), (see also Fig. 6b).

We interpret x and y as being fragments of α' which have been cleaved by trypsin because of the following data.

(i) Tryptic digestion of electrophoretically purified ATPase T_a produced a protein complex which cannot be distinguished from the T_i so far examined in terms of electrophoretic mobility on 5% polyacrylamide gels, sodium dodecyl sulfate disc-electrophoresis pattern and lack of enzyme activity.

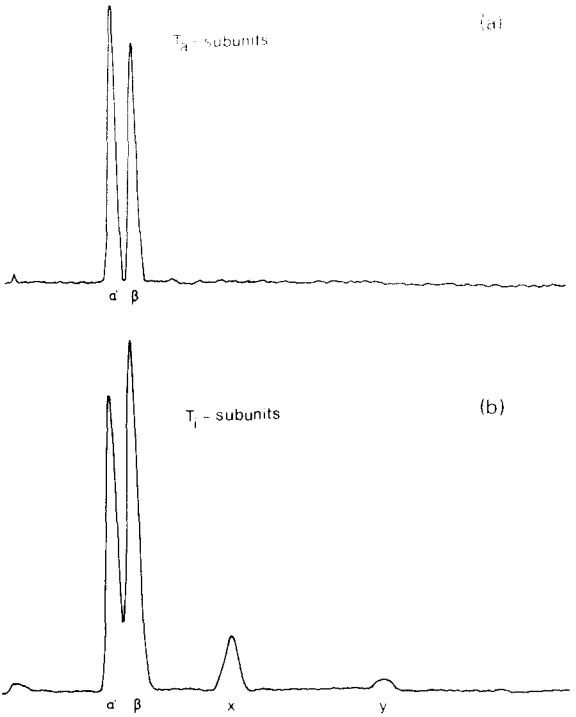

Fig. 6. (a) ATPase T_a (30 μg protein) sodium dodecyl sulfate electrophoresis on 7.5% polyacrylamide. Gels were scanned using a Gilford spectrophotometer. (b) ATPase T_i (60 μg protein), see (a).

(ii) The sum of molecular weights of x and y is in good agreement with the molecular weight of α'.

(iii) The loss of staining intensity of α', when T_a is converted to T_i by tryptic digestion, referred to as β, whose staining intensity does not change, is compensated by the sum of x and y staining intensities (Table I).

Trypsin treatment of Me^{2+}-(13 S) ATPase has been carried out with preparations from chloroplasts [30] and bacteria [14] to release "latent" ATPase activity.

Structural aspects of the effect of trypsin have been reported for (13 S) ATPases from *E. coli* [19,21] and *S. typhimurium* [21]. The present results from trypsin treatment of (13 S) ATPase from *Micrococcus* sp. ATCC 398E are a first approach to the elucidation of the relevance of certain structural features

TABLE I

RELATIVE STAINING INTENSITIES OF SUBUNITS OF ATPase T_a AND T_i ON SODIUM DODECYL SULFATE POLYACRYLAMIDE GELS (β = 1.00)

	T_a	T_i	M_R
α'	1.19 ± 0.03	0.78 ± 0.03	61 000
β	1	1	55 000
x	—	0,30 ± 0.06	38 000
y	—	0.06 ± 0.01	22 000

to the enzymic function. The shortening of the α-chains and the removal of γ-subunit with ATPase T_a retained the activity indicating that α-, even partly degraded, and β-subunits are sufficient for ATP hydrolysis.

Similar conclusions have been drawn for ATPase from *M. lysodeikticus* by Salton et al. [12], for ATPase from *E. coli* by Bragg and Hou [21] and Nelson et al. [19], for ATPase from *B. megaterium* by Mirsky and Barlow [17], and for ATPase from mitochondria by Kozlov and Mikelsaar [8]. In contrast to these authors, Kobayashi and Anraku [20] have stated that the γ-subunit of *E. coli* ATPase is needed for hydrolytic activity. In view of our results we see the need for more detailed investigations to settle this discrepancy. To our knowledge the shortening of the α-chain (M_R 65 000), which yields the α′-chain (M_R 61 000), has not yet been reported for bacterial and related ATPases. We assume the presence of an exposed region, which is easily attacked by proteases inasmuch as chymotrypsin, bacterial extracts, and impurities in DNAase purity grade II cause similar results, whereas DNAase purity grade I shows no effect.

Preliminary experiments with trypsin at concentrations lower than those described here and shorter times of incubation have shown that fraction N (the untreated ATPase complex) is converted into fraction T_a via at least two intermediates which have electrophoretic mobilities between those of N and T_a. These intermediates probably represent modified ATPases, in which one or two α-chains have been converted into α′-chains and/or in which the γ-chain has been removed.

In the case of T_i, an additional conversion of α′-chain(s) into x- and y-fragments appears to have occurred. This step results in a complete loss of ATP-hydrolyzing activity although the ability to bind ATP is retained. These results indicate that a certain quaternary structure of α′- and β-subunits is necessary to hydrolyze ATP although it is not necessary for ATP binding. A more detailed characterization of the T_i complex in comparison with the enzymatically active ATPase complexes will help to distinguish between those functional groups which are needed for ATP binding and ATP hydrolysis.

Besides ATPase T_i, no other high molecular weight protein complexes could be discovered after prolonged trypsin treatment of ATPase T_a. This could either occur because ATPase T_i is relatively resistant to further trypsin degradation or because the products of trypsin degradation disintegrate quickly into smaller fragments which appear only as front bands in 5% polyacrylamide gel electrophoresis.

The analysis of the staining intensities of the bands obtained by sodium dodecyl sulfate gel electrophoresis indicates that a third of the α′ is cleaved into x- and y-fragments during conversion of ATPase T_a into T_i (Table I). With regard to the molecular weights of the subunits and the whole enzyme, a third of the α′-chains would correspond to one out of three α′-chains per T_a-complex. Thus, our results support the subunit composition of $\alpha_3\beta_3\gamma(\delta\epsilon)$ as proposed for Me^{2+}-(13 S) ATPases from different origins [6,14,21,36]. The question of subunits beyond the α-, β-, and γ-components is so far not settled. Subunits γ and δ seem to involve the problem of binding to and/or coupling with other proteins associated with membrane structure and electron transfer [11,18,30].

Subunit ε has been regarded as the natural ATPase inhibitor protein in the case of chloroplast ATPase [30]. Our purified (13 S) ATPase from *Micrococcus* sp.

ATCC 398E, however, lacks the δ- and ε-components. More work is under way in this laboratory concerning the binding of ATP and ADP, their mutual conversion and the coupling of these functions to the respiratory chain.

Acknowledgements

This work was supported in part by Deutsche Forschungsgemeinschaft, grant Ri 268/1 and Do 192/1. We are indebted to Professor P. Flesch who advised us in culturing the microorganisms and placed his fermenter at our disposal (DFG Fl 290/1a). We also wish to thank Dr. Karin Neunhoeffer for helping us in culturing the microorganism and Miss Christel Brand for revising the manuscript.

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