REVISTA ESPAÑOLA DE FISIOLOGIA, 34, 309-316. 1978

Effect of Group Specific Reagents on the $Mg^{2\pm}$ Dependent Activity of Purified *Micrococcus lysodeikticus* ATPase *

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(Received on November 30, 1977)

J. CARREIRA, K. DOSE and E. MUÑOZ. *Effect of Group Specific Reagents on the Mg^{2+}-Dependent Activity of Purified* Micrococcus lysodeikticus *ATPase.* Rev. esp. Fisiol., 34, 309-316. 1978.

A series of group specific reagents has been examined for their ability to inactivate *Micrococcus lysodeikticus* adenosine triphosphatase assayed with Mg^{2+} as activating divalent cation. The enzyme activity was not inhibited by sulphydryl, carboxyl, histidine, arginine and methionine specific reagents at inhibitor concentrations below 2 mM. However, the ATPase was inactivated by its chemical reaction with either one molecule of trinitrobenzenesulfonic acid or tetranitromethane, or two to four molecules of N-bromosuccinimide. These results suggest that at least one amino group, one tyrosine and two to four tryptophans are involved in the Mg^{2+}-dependent binding or hydrolysis of ATP.

The ATPase of *Micrococcus lysodeikticus* has been purified and characterized (3). It has a molecular weight of 345,000 and consists of two major subunits of molecular weight about 50,000 and minor ones with molecular weights ranging from 10,000 to 41,500. These properties are in general similar to those of mitochondrial (7), chloroplast (23), *Escherichia coli* (6) and *Streptococcus faecalis* (1) ATPases but differ to some extent from those of some gram positive bacteria (21) with regard to the minor subunits.

Like mitochondrial (29), chloroplast (23) and *E. coli* ATPases (9, 25), *Micrococcus lysodeikticus* ATPase presents the phenomenon of latency in its activity (22). It seems likely that this phenomenon is due to the regulatory effect of a small subunit (ε) of the enzyme which modulates

* This investigation was supported by grants from the «Deutscher Akademischer Austauschdienst» (DAAD) and «Fondo Nacional para la Investigación Científica».

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the hydrolytic activity of the ATPase (11). This natural inhibitor would control the hydrolysis of the ATP formed in respiration (22, 34).

Although the hydrolytic activity of the ATPase may not be the main function of the enzyme, at least in aerobic organisms, the study of this activity has provided most of the existing knowledge about ATPase functional properties (26). Experiments to determine the structure of the active site of mitochondrial ATPase proved that no sulphydryl group was involved in the binding of ATP to ATPase (30) and that the chemical modification of one tyrosine residue per ATPase molecule inhibited completely the hydrolytic activity of the enzyme (12).

This report presents evidence indicating that at least three amino acyl residues have an important role in the hydrolysis of ATP by *M. lysodeikticus* ATPase. One amino group, one tyrosine and two to four tryptophans per enzyme molecule appear to be involved in the formation of the active or regulatory sites, or related to them in such a way that the chemical modification of any of those residues causes the inactivation of the ATPase. We have used Mg^{2+} as activating cation to facilitate comparisons with membrane-bound ATPase which showed a higher Mg^{2+} — than Ca^{+2} — dependent activity (22).

Materials and Methods

The ATPase of *Micrococcus lysodeikticus* used in these experiments was purified by preparative electrophoresis as described elsewhere (4).

The reagents were prepared daily in the buffers employed in the preincubation medium. Some of them were dissolved in organic solvents, with controls to test the effect of the solvent on the enzyme activity.

The experiments on the inactivation of the ATPase by chemical modification of specific residues with different reagents were carried out as follows: 5 μg of purified ATPase were preincubated at 30° C with inhibitor concentrations ranging from 3 μM to 2 mM. The preincubation times varied from 30 minutes to 3 hours and the final volume was 500 μl. Some reagents were tested at different pH values in order to operate on the region of their maximum reactivity. The buffers (50 or 100 mM) used were acetic acid-sodium acetate (pHs 4.9, 5.0, 5.2), cacodylate-NaOH (pHs 6, 6.5, 6.8, 1), Tris-maletate (pHs 8, 8.5, 8.7), Tris-HCl (pHs 7.5, 7.9, 8, 8.5, 8.9), sodium bicarbonate-NaOH (pHs 8.5, 9.5) and glycine-NaOH (pHs 9.5, 10). At the end to the preincubation time 4.45 ml of 50 mM Tris-maleate pH 7.6 at 35° C, were added to each tube and 1 min later the reaction medium was completed with 50 μl of 100 mM Mg-ATP. The hydrolysis of ATP was followed during 5 min at 35° C using the method described by ARNOLD *et al.* (5). Controls were made by preincubating the enzyme in absence of the inhibitor. It is worth noting that the specific activities of these controls varied somewhat depending on the pH of preincubation, specially at pH values below 6.5.

To test whether the presence of ATP and Mg^{2+} could alter the effect of the reagents on the ATPase activity, all the preincubation experiments were performed in four different ways: presence of 1 mM ATP, presence of 1 mM Mg^{2+}, presence of 1 mM Mg-ATP and absence of both substances.

Protein was determined by the method of LOWRY *et al.* (17) or calculated from the $E^{1\%}_{1\,cm} = 6.9$ at 276 nm as previously estimated (24).

The number of inhibitor molecules bound per molecule of enzyme was calculated according to the method used by TAKETA and POGELL (33) for inhibitors which bind reversibly to the enzymes.

The reagents employed for the different specific groups were as follows. Amino groups: trinitrobenzenosulfonic acid (Ser-

va Feinbiochemica) at pHs 8.0, 8.5 and 9.5: 2-methoxy-5-nitropone from Serva at pH 8.5: *O*-methylisourea (Serva) at pH 9.5 and 10.0 and naphthoquinone sulfonic acid (Merck) at pM 8.5 and 9.5. Carboxyl groups: 1-cyclohexyl-3-(2-morpholinyl-4-ethyl) carbodimide metho *p*-toluenesulfonate from Aldrich at pHs 4.9, 5.0, 5.2, 6.0 and 7.0; 1-ethyl-3-(3-dimethylaminopropyl) carbodimide (EGA-Chemie KG) at pH 5.0, and triethyloxonium fluoroborate (Fluka AG), at pH 5.0. Both, 1-cyclohexyl-3-(2-morphonyl-4-ethyl) carbodiimide metho *p*-toluenesulfonate and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide were used with taurine and glycine methyl ester as a nucleophilic agents. Sulphydryl groups: 5,5′-dithiobis (2-nitrobenzoic acid) from Calbiochem at pHs 6.8, 8.0, 8.5 and 8.9; *p*-chloromercuribenzoic acid from Serva at pHs 8.0, 8.5 and 8.9, and *N*-ethylamaleiimide from Fluka at pH 6.0, 6.8, 7.0 and 7.9. Tyrosine: Tetranitromethane from Fluka AG at pH 7.5. Arginine: glyoxal at pH 8.5 and phenylglyoxal (both from Fluka) at pH 7.5, and 2-hydroxyl-5-nitrobenzyl bromide from Merck at pH 7.0. Histidine: bromoacetone prepared by the method of LEVENE (16) at pH 6.5 and 7.0. Methionie: ω-bromoacetophenone from Merck at pH 7.5 and 8.5.

Results

Under the described conditions the ATPase activity could not be altered when the enzyme was preincubated in the presence of reagents for carboxyl, sulphydryl, arginine, histidine and methionie residues. The addition of either ATP, Mg^{2+} or both did not produce any further effect.

Figure 1 shows the inhibion of ATPase by trinitrobenzenesulfonic acid when the preincubation was carried out at pH 8.0. Higher pH values did not increase the percent of inactivation. The Ki for trinitrobenzenesulfonic acid was 0.85 mM. The presence of Mg^{2+} in the preincubation medium apparently sensitized the ATPase

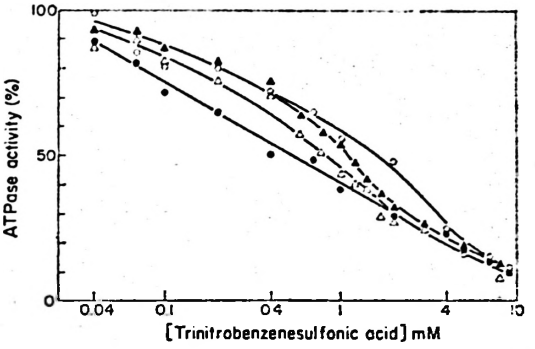

Fig. 1. *Inactivation of the purified ATPase of* M. lysodeikticus *by trinitrobenzenesulfonic acid.*

The preincubation time with the inhibitor was 1 hour and longer times did not increase the effect of the inhibitor. The ATPase activity is presented as % of remaining activity. ▲—▲, Preincubation in presence of 1 mM ATP; O—O, in presence of 1 mM Mg-ATP; Δ—Δ, in absence of both; ●—●, in presence of 1 mM Mg^{2+}. Other conditions as described in Methods.

to reagent (Ki ≃ 0.5 mM) while ATP protected the enzyme (Ki ≃ 1.02) but the experimental evidence was not sufficient to conclude clearly on the influence of these effectors.

Naphthoquinone sulfonic acid at pH 8.5 inhibited the ATPase in a similar way to the inhibition by trinitrobenzenesulfonic acid *(Results not shown)* but 2-methoxy-5-nitrotropone and *O*-methylisourea had no significant effect on the enzyme activity.

Figure 2 illustrates the inactivation of ATPase with tetranitromethane at pH 8.0. In this case, it was evident that ATP protected the ATPase. However, the apparent sensitizing effect of the cation was again little marked. From the inhibition data, log (Vo-v)/v was plotted *versus* log [inhibitor]. A straigth line was obtained whose slope *n* gave the apparent number of inhibitor molecules reacting per enzyme molecule to inactivate it, assuming one active site per enzyme molecule (see 33 and Materials and Methods). The slope was close to 1 ($n = 0.9$) suggesting the

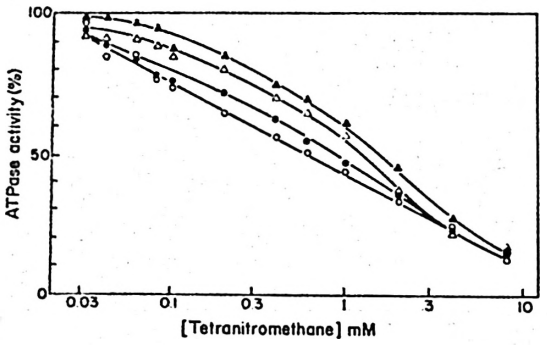

Fig. 2. *Inactivation of ATPase with tetranitromethane.*
The reagent was dissolved in ethanol and added to the medium in a small volume so that the ethanol had no effect on the enzyme activity. The preincubation time was 1 hour and carried out under different conditions: ▲—▲, presence of 1 mM ATP; △—△, presence of 1 mM Mg-ATP; ●—●, absence of both; ○—○, presence of 1 mM Mg^{2+}.

modification of one single residue. Under our experimental conditions, tetranitromethane would react selectively with tyrosine in absence of cysteine (32). The lack of ATPase inhibition by specific sulphydryl group reagents together with the low content of cysteine i.e., less than one mol per mol of subunit in this ATPase (1), points to such a selective action on one tyrosine residue. Although it cannot be ruled out that sulphydryl group (s) may be involved in the active site, sensitized by a prior reaction of a tyrosine with tetranitromethane, the quantitative results (see above) argue against this possibility.

Figure 3 shows the inhibition of the ATPase with *N*-bromosuccinimide at pH 7.5. ATP added to the preincubation medium slightly protected the enzyme from its inactivation. The protection only occurred at very low concentrations of *N*-bromosuccinimide, the Ki being 14 μM. The *N*-bromosuccinimide concentration that inhibited 50 % of ATPase activity was 12 μM in all the other cases. *N*-bromosuccinimide is known to react with tryptophan and sulphydryl groups and also with tyrosines and histidines although the reactivity with the last two amino acyl residues is much lower (31). The mild preincubation conditions used (pH 7.5, room temperature) were optimum for the modification of tryptophan residues in absence of SH groups (31). Therefore, the very low concentrations of inhibitor

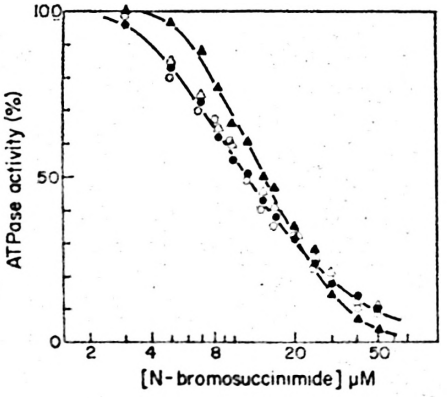

Fig. 3. *Inactivation of ATPase with* N-*bromosuccinimide.*
The preincubation time was 1 hour in 0.1 M Tris-HCl, pH 7.5: ▲–▲, preincubation in presence of 1 mM ATP; ●–●, in presence of 1 mM Mg^{2+}; △—△, in presence of 1 mM Mg-ATP; ○—○, in absence of both.

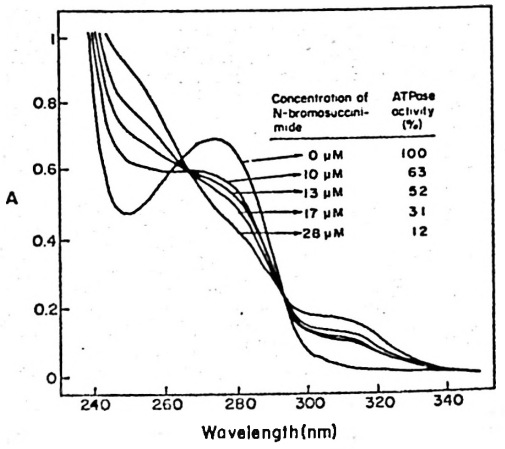

Fig. 4. *Correlation between Inactivation of ATPase with* N-*bromosuccinimide and change in the absorption spectrum of the enzyme.* The preincubation conditions were the same as in figure 3, except that ATP and Mg^{2+} were absent. Concentrations of *N*-bromosuccinimide and the % of residual ATPase activity are indicated in the figure.

that inactivated the ATPase suggested that its inhibition resulted from the modification of tryptophan residues, since the involvement of sulphydryl groups was unlikely (see above). From a plot of log (Vo-v)/v *versus* log [*N*-bromosuccinimide], two straigth lines were obtained whose slope values were 1.74 and 3.5 respectively indicating that two to four tryptophans were modified per enzyme molecule.

The correlation between enzyme inactivation and change in the absorption spectrum of the ATPase following its preincubation with *N*-bromosuccinimide is illustrated in figure 4. As the *N*-bromosuccinimide concentration increased from zero to 28 μM the ATPase activity decreased from 100 % to 12 %. This paralleled the decrease in absorption at 280 nm. After this treatment with *N*-bromosuccinimide, the tryptophan content of the ATPase was determined as already reported (24). A value of 16 ± 2 mol tryptophan per mol of ATPase (350,000 g) was found in that case instead of the 22 ± 2 mol value previously estimated for the native protein (24).

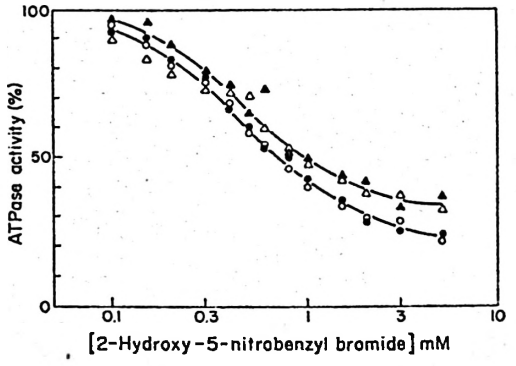

Fig. 5. *Inactivation of ATPase with 2-hydroxy-5-nitrobenzyl bromide.*
The enzyme wa preincubated with the inhibitor at room temperature, during 30 min and in 100 mM cacodylate-NaOH. The 2-hydroxy-5-nitrobenzyl bromide was dissolved in acetone and used in a very small volume, so that the solvent did not affect the activity of ATPase; ▲—▲, preincubation in presence of 1 mM ATP; △—△, presence of 1 mM Mg-ATP; O—O, presence of 1 mM Mg^{++}; ●—●, absence of both. Preincubation times of 15 minutes gave the same results.

Figure 5 represents the inactivation of the ATPase by the KOSHLAND'S reagent, 2-hydroxy-5-nitrobenzyl bromide (15). The preincubation was carried out at pH 7, room temperature for 30 min. The apparent Ki was about 0.7 mM. However, since the «half-life of 2-hydroxy-5-nitrobenzyl-bromide in aqueous solutions is less than 1 minute» (20) it can be assumed that the actual Ki was many times lower. The specificity of 2-hydroxy-5-nitrobenzyl bromide for tryptophan is well known (15).

Discussion

The present work has provided some information about the area of the hydrolytic site of the Mg^{+2}-dependent ATPase of *Micrococcus lysodeikticus*.

The lack of inhibition of the ATPase by reagents of carboxyl, sulphydryl, arginine, histidine and methionine residues suggests either that these residues are absent from the active site of the enzyme or are not accessible to the reagents. Similar results, as far as the sulphydryl groups are concerned, were obtained with rat liver (27) and beef heart (30) mitochondrial ATPase as well as with chloroplast coupling factor 1 (8). However, in this last case a role of sulphydryl groups for the photophosphorylating activity of the protein was suggested (19). In a recent study, AHLERS *et al.* (2) reported on the inhibition of *E. coli* ATPase by SH reagents but from the complex results obtained, they concluded that these groups were probably not directly located in the active center. It is worth noting that MARCUS *et al.* (18) have very recently described the inhibition of mitochondrial ATPase by chemical modification of arginine residues. These authors used higher concentrations of the reagents than in the present

experiments. This may account for the discrepancies.

In agreement with previous results on *E. coli* ATPase (10) the membrane bound ATPase of *M. lysodeikticus (Results not shown)* was inhibited by the water soluble carbodiimides even in absence of nucleophilic agents while the purified enzyme was not sensitive to the same reagent. As a matter of speculation it might be suggested that the enzyme bound to the membrane has a conformation that facilitates the access of reagents to the carboxyl groups or that proteins other than ATPase participate in the inactivation mechanism. The last notion appears to be supported by the recent work by FILLINGAME (13) who purified the carbodiimide-reactive protein from *E. coli* ATPase and identified it with a proteolipid of molecular weight 8,4000.

The role of amino residues in the hydrolytic function of purified *M. lysodeikticus* ATPase as suggested by the inhibition of the enzyme with trinitrobenzenesulfonic acid agrees with previous work on mitochondrial (18) and *E. coli* ATPases (2). It is worth noting that trinitrobenzenesulfonic acid may also react with the imidazol moiety of histidine residues. However this possibility seems very unlikely in *M. lysodeikticus* ATPase because of the lack of inhibition of enzymic activity by bromoacetone and photochemical oxidation, which behave as specific reagents for histidines. It is well known that trinitrobenzenesulfonic acid reacts with nonprotonaded amino groups (14). The pK of α-amino groups is generally lower than that of ϵ-amino groups. The similar reactivity shown by *M. lysodeikticus* ATPase towards this reagent at pH values of 8.0 and 9.5 may then suggest than an α-amino group rather than an ϵ-amino group is involved in the effect of this reagent. The same suggestion is also supported by the negative result obtained with *O*-methylisourea. This reagent shows a higher reactivity for ϵ-amino groups (20) when assayed at pH 9.5-10.0, but no inhibition of the ATPase was observed under these conditions. Nevertheless, it is obvious that the elucidation of the problem must await further experimentation.

The involvement of tyrosine residues in binding or hydrolysis of ATP of mitochondrial ATPase was described firstly by PENEFSKY (28) who inhibited the enzyme activity with iodine in presence of 4 mM ATP. SENIOR (30) made use of tetranitromethane and concluded that the ATPase inhibition was due the chemical modification of tyrosine instead of sulphydryl groups, since the latter were not affected by SH group reagents. FERGUSON *et al.* (12) showed that the modification of one molecule of tyrosine per molecule of mitochondrial ATPase was responsible for the loss of the enzyme activity.

The present data agree quite well with the previously reported findings. Although the *N*-acetylimidazole failed to inhibit the ATPase activity, the results with tetranitromethane show that at least one molecule of tyrosine must be closely related to the binding or hydrolysis of ATP by the ATPase of *M. lysodeikticus*. A value of about 22 tryptophan residues per molecule of *M. lysodeikticus* ATPase has been well established (24). Two to four tryptophans of each ATPase are likely to be involved in the hydrolytic activity to the enzyme. Two possibilities can be suggested to explain this function of tryptophans. First, the amino acyl residues may be part of the active centre of the ATPase. Second, the integrity of those tryptophans is essential to keep the right conformation of the active centre, although they may not participate in the structure of the active site.

Finally another aspect deserves some comment. On the basis of the present results, the opposite effects of ATP and Mg^{2+} might be considered as a consequence of the different conformations of the enzyme induced by the nucleotide or the cation.

Acknowledgements

The authors are indebted to Dr. H. U. Wolf for the generous supply of the reagents and constant advice in their use and to S. Risi for helpful discussions.

Resumen

Se ha examinado la capacidad de una serie de reactivos específicos de grupo para inactivar la adenosina trifosfatasa de *Micrococcus lysodeikticus* ensayada en presencia de Mg^{2+} como catión divalente activador. La actividad enzimática no se inhibía con reactivos específicos de grupos sulfhidrilo, carboxilo, histidina, arginina y metionina cuando éstos se utilizaban a concentraciones inferiores a 2 mM. Sin embargo, la ATPasa se inactivaba por su reacción química con una molécula de ácido trinitrobencesulfónico y tetranitrometano, o con dos-cuatro de *N*-bromosuccinimida. Estos resultados sugieren que al menos un grupo amino, una tirosina y dos a cuatro triptófanos están implicados en la fijación o en la hidrólisis de ATP dependiente de Mg^{2+}.

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