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PROPERTIES OF MEMBRANE-BOUND ADENOSINE TRIPHOSPHATASE FROM *XANTHOMONAS ORYZAE*[1,2]

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Abstract

A membrane-bound adenosine triphosphatase was isolated from *Xanthomonas oryzae*. The enzyme required Mg^{++} for its activity. The Mg^{++}-activation curve gives an optimal ratio of Mg^{++}/ATP of 1.5. Mg^{++} is not only required for its activity but also required for binding of the enzyme to membrane and for the stability of the enzyme itself. The enzyme hydrolyzed nucleoside triphosphates and diphosphates, but not monophosphates. The optimal pH for activity is 7.5 and the optimal temperature is 50°C.

Introduction

Previous report showed a non-specific ATP hydrolytic enzyme located on the surface of the cell wall of *Xanthomonas oryzae*. The enzyme was extensively purified and identified as nucleotidase (Huang *et al.*, 1973). Later it was found that the enzyme attacked not only nucleotides but also primary phosphoryl group of many other phosphomonoesters, therefore, the enzyme was revised to be phosphatase (Huang *et al.*, 1975). After bacterial cell walls were removed by lysozyme, the bacterial cells rapidly converted to spheroplasts and a large amount of phosphatase released out. When spheroplasts were broken and a membrane fraction was harvested a ATP hydrolytic enzyme reguiring Mg^{++} was detceted, the enzyme appears to be a membrane-bound adenosinetriphosphatase (ATPase-EC 3. 6. 1. 3.). Membrane-bound ATPase from bacterial system reported in the past usually has a broad specificity toward nucleotides. The ATPase from *Lactobacillus arabinosus* (Cole and Hughes, 1965), and the Mg^{++}-activated ATPase from *E. coli* K-12 (Hafkenscheid and Bonting, 1967) hydrolyze ATP, ADP and other nucleoside triphosphates.

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Other ATPases, such as that from *Staphylococcus aureus* (Gross and Coles, 1968), *Vibrio parahaemolyticu* (Hayashi and Uchida, 1965) and *Streptococcus facalis* (Abrams and Baron, 1967) were able to hydrolyze some of the nucleoside triphosphates besides ATP. In these experiments, the term ATPase used was referred to the enzyme having the best activity of cleaving the terminal phosphate group from ATP with no consideration to the amount of phosphorus released per molecule of nucleoside triphosphate. The sense has generally been used for the membrane-bound ATP hydrolytic enzyme in bacterial system.

Membrane-bound ATPase in bacterial system has been reported in *Escherichia* (Voelz 1964), *Bacillus* (Weibull *et al.*, 1962), *Micrococcus* (Ishikawa and Lehninger, 1962; Muñoz *et al.* 1969). *Streptococcus* (Abrams *et al.* 1960), *Staphylococcus* (Gross and Coles, 1968), *Pseudomonas* (Drapeau and Maclead, 1963), *Lactobacillus* (Cole and Hughes, 1965), *Vibrio* (Hagashi and Uchida, 1965), and *Agrobacterium* (Gainor and Phillips, 1969). But no information concerning the ATPase from *Xanthomonas* was reported. In this investigation a membrane-bound ATPase from *X. oryzae* was isolated, partially purified and its properties studied.

Materials and Methods

Organism

Xanthomonas oryzae 507, a mutant of *Xanthomonas oryzae* 500 preserved at this laboratory was used. This organism contains less capsule and is more sensitive to lysozyme than wild type.

Chemicals

Nucleotides, lysozyme, bovine serum albumin, tris and EDTA were purchased from Sigma Chemical Co. Inorganic chemicals were of reagent grade.

Medium

The medium used was potato-peptone medium containing potato, 200 g; peptone, 5 g; $Ca(NO_3)_2 \cdot 4H_2O$, 0.5 g; $Na_2HPO_4 \cdot 12H_2O$, 2 g; sucrose, 15 g; and water 1000 ml.

Growth of bacteria

The bacteria grown in slant was transfered to potato-peptone medium and incubated at 30°C on a rotatory shaker for 24 hours. The bacteria was harvasted and washed twice with 0.01 M tris buffer at pH 7.5 and finally suspended in the same buffer.

Isolation and partial purification of the membrane-bound ATPase

The washed bacteria were suspended in 0.01 M tris, pH 7.5 in the presence of 20% sucrose and 0.01 M EDTA, and then 0.16 mg per ml of lysozyme was added and incubated at 30°C for 40 min. At the end of incubation, 99% of bacterial cell became spheroplast. The spheroplasts were precipitated by

centrifugation at 1000 g for 10 minutes. The precipitated spheroplasts were transfered to 0.01 M tris buffer in the presence of 0.01 M $MgCl_2$ for "osmotic shock", then DNAse (2 mg/100 ml) and RNAse (2 mg/100 ml) were added and incubated at room temperature for one hour. The membrane was precipitated by the centrifugation at 23500 g for 10 minutes. The membrane was washed 5 times with the same buffer solution and then transfered to 0.0001 M tris buffer pH 7.5 in the absence of $MgCl_2$ at 4°C for 10 hours with gentle stirring. Under these treatments ATPase released from the membrane. The membrane was removed by centrifugation and the supernatant containing ATPase was subjected for fractionation by adding different concentration of ammonium sulfate. The enzymes precipitated from 30 to 50% of ammonium sulfate saturation was collected and dialysis against tris buffer. The ATPase prepared by these procedures was free from non-specific phosphatase. This preparation was used as the enzyme source for the characterization of ATPase.

Assay for ATPase

ATPase was assayed by the liberation of inorganic orthophosphate from ATP. Reaction mixture contained 0.005 M disodium ATP, 0.005 M $MgCl_2$, 0.05 M tris buffer at pH 7.5 and proper amount of enzyme preparation in a final volume of 1 ml. The reaction was carried out by incubating the reaction mixture at 37° for 10 min.. The reaction was stopped by adding 0.1 ml of 25% TCA. The release of Pi was measured according to the procedures described by Fiske and Subbarow (1925). One unit of enzyme activity was defined as the amount of enzyme able to liberate 1 μ mole of Pi per 10 min. at 37°.

Protein determination

Protein was measured according to the method described by Lowry *et al.* (1951) with bovine serum albumin as standard.

Results

Isolation and purity of ATPase

The membrane isolated from *Xanthomonas oryzae* contained a ATPase which could be released from the membrane in low ionic strength solution in the absence of multivalent cations. The crude enzyme preparation from the membrane usually contaminated with non-specific phosphatase. Since any non-specific phosphatase also acts on the ATP in this experiment, the non-specific phosphatase activity was estimated concurrently by using phenylphosphate as a substrate. The assay condition was similar as described for ATPase except that there was no presence of Mg^{++}. From the difference between the ATP hydrolysis and the phenylphosphate hydrolysis gave the approximate value of specific ATPase activity. As shown in Table 1, ATPase released by "cold

Table 1. *Procedures for the releasing of ATPase from the membrane of Xanthomonas oryzae and its partial purification.*

Procedures	Results
1. Lysozyme-EDTA Treatment	Spheroplast
2. Osmotic shock	"Membrane ghost"
3. Washing 1	Releasing of non-specific phosphatase and other proteins.
Washing 2	Releasing of non-specific phosphatase and other proteins.
Washing 3	Releasing of non-specific phosphatase and other proteins.
Washing 4	Releasing of non-specific phosphatase and other proteins.
Washing 5	No further releasing.
4. "Cold shock" in the absence of Mg^{++}.	Releasing of ATPase and other proteins.
5. Precipitated with ammomium sulfate (30 to 50%)	Free from non-specific phosphatase.

shock" was contaminated with small amount of non-specific phosphatase. The contamination could be eliminated by fractionation with different concentration of ammonium sulfate. The fraction precipitated from 30 to 50% of ammonium sulfate was free from the contamination of non-specific phosphatase. The yield of ATPase from these fractions was 60 to 70%. The purity of ATP hydrolitic activity was examined directly by polyacrylamide gel electrophoresis as described before (Huang *et al.* 1973). Only one single active band for ATP hydrolysis was obtained.

Effect of Mg^{++} on the release of ATPase

Mg^{++} was required for the binding of ATPase to membrane. In the absence of Mg^{++}, ATPase released from the membrane. If Mg^{++} was added (0.01 M) after ATPase was released, about 30% of the released ATPase could be restored to membrane. The result indicated that once the enzyme was dissociated into solution the enzyme could not all reassociate with membrane by addition of Mg^{++}.

Effect of various cations on ATPase activity

Various cations were used to test their effect on enzyme activity. As shown in Table 2, among the cations tested only Mg^{++} showed important effect on enzyme activity. Mn^{++}, Ca^{++}, Na^{+}, or K^{+} added alone or with Mg^{++} ion did not have significant stimulation effect on ATPase activity. The effect of the concentration of Mg^{++} on the activity of ATPase is shown in Fig. 1, The optimal Mg^{++}/ATP ratio was 1.5.

Table 2. *Effect of various cations on ATPase activity.*

All cations are chloride salts. 5×10^{-3} M salt free ATP was used in this experiment. The concentration of cations added was equal to the amount of ATP used. The enzyme activity in the presence of Mg^{++} was considered as 100%.

Cation added (5×10^{-3} M)	Activity (%)
None	11
Na^{+}	10
K^{+}	9
Ca^{++}	13
Mn^{++}	15
Mg^{++}	100

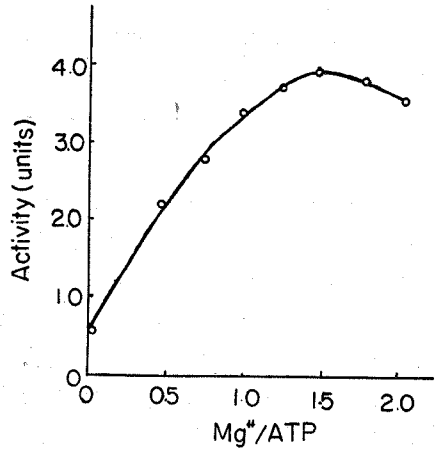

Fig. 1. Activation of ATPase by Mg^{++}. ATP concentration was 0.005 M. Reaction was processed in 0.05 M tris buffer, PH 7.5.

Table 3. *Substrate specificity for membrane-bound ATPase from Xanthomonas oryzae.*

The concentration of all substrates were at 5×10^{-3} M. The percentage of activity is based on 100% for ATP.

Substrate	Activity (%)
ATP	100
GTP	72
CTP	75
UTP	83
ADP	86
CDP	60
UDP	70
XMP	0

Substrate specificity

Various nucleotides were tested for the substrate specificity of ATPase. As shown in Table 3, the enzyme was active against nucleoside diphosphates and triphosphates but not monophosphates.

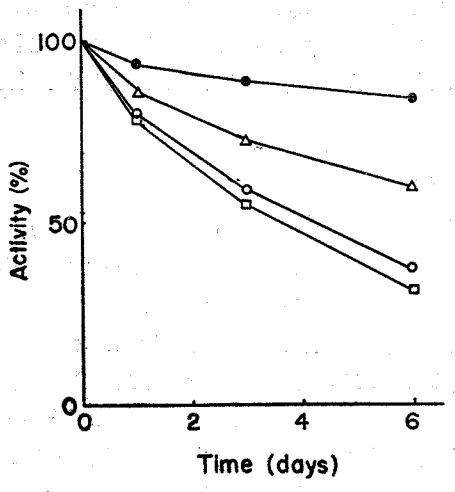

Fig. 2. Effect of storage on the stability of ATPase. The enzyme was stored at 4°C in following solutions. □—□: 0.01 M tris buffer, pH 7.5; ○—○: 0.01 M tris buffer, pH 7.5 in the presence of 20% glycerol; △—△: 0.01 M tris buffer, pH 7.5 in the presence of 20% alcohol; ●—●: 0.01 M tris buffer, pH 7.5 in the presence of 5×10^{-3} M $MgCl_2$.

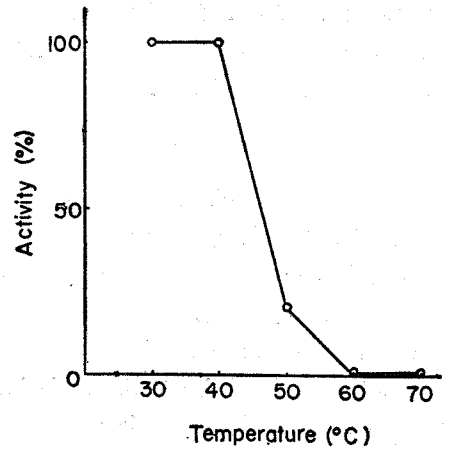

Fig. 3. Effect of heating on the stability of ATPase. Enzyme in 0.05 M tris buffer, pH 7.5 was incubated at various temperature for 10 min before its activity was assayed. Enzyme activity at 30°C was considered as 100%.

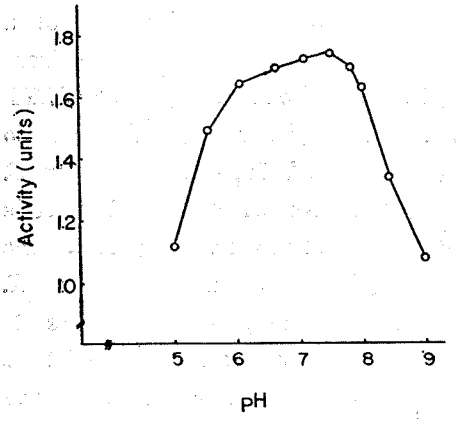

Fig. 4. Effect of pH on the hydyolysis of ATP. Buffer were prepared by adjusting 0.05 M tris-buffer to the desired pH with acetic acid at 37°C.

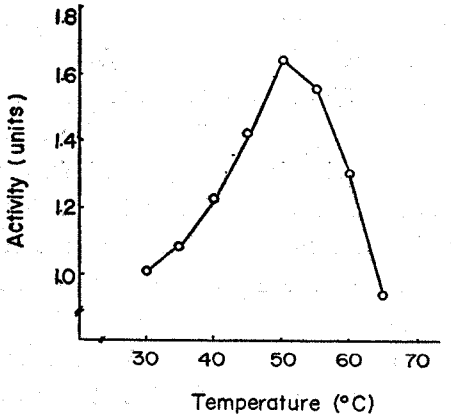

Fig. 5. Effect of temperature on the activity of ATPase. The reaction mixtures were placed in test tube and incubated in various temperature for 10 min.

Enzyme stability and effects of pH and temperature

ATPase was very unstable during storage. When the enzyme was stored in 0.01 M tris buffer, pH 7.5, at 4°C for 6 days, 70% of activity was lost, however, when 0.005 M Mg^{++} was supplied only 15% of activity was lost (Fig. 2). The enzyme was also unstable to beating. When ATPase was incubated at 50°C, for 10 minutes, 80% of activity was lost (Fig. 3). The enzyme has an optimal pH at 7.5 (Fig. 4) and temperature at 50° for its enzyme activity (Fig. 5).

Discussion

After Post *et al.* (1960) reported a (Na^+-K^+)-dependent and ouabain sensitive ATPase activity in broken erythrocyte membrane, several (Na^+-K^+)-activated ATPases have been described in several varieties of animal tissues (Bonting *et al.*, 1961). In bacteria, similar type of ATPase has been reported in *Staphylococcus aureus*, (Gross and Coles, 1968), *Streptococcus faecalis* (Abrams *et al.* 1960) and *Vibrio parahaemolytieus* (Hagashi and Uchida, 1965), the ATPases isolated from these bacteria are activated by Mg^{++} and stimulated by K^+ or Na^+, however most of ATPases from other bacteria require only divalent cation as its activator. For example, ATPase from *Bacillus megaterium* or *Micrococcus lysodeikticus* is activated by Mg^{++} or Ca^{++}; ATPase from *Lactobacillus arabinosus* is activated by Mg^{++} only. The ATPase from *Xanthomonas oryzae* is similar to the ATPase from *Lactobacllus arabinosus*, only Mg^{++} is required.

Gross and Coles (1968) demonstrated that the Mg^{++}/ATP ratio of the ATPase from *Staphylococcus aureus* is 1.0. Based on this value they suggested that one Mg^{++} is associated with one ATP and whole complex acts as a substrate. The same Mg^{++}/ATP ratio was also reported on the ATPase from *Lactobacillus arabinosus* (Cole and Hughes, 1965). However, the Mg^{++}/ATP ratio deviated from 1.0 was also reported. For example, the Mg^{++}/ATP ratio for that of *Micrococcus lysodeikticus* is 0.5 (Muñoz *et al.*, 1969) and 0.4 for *E. coli* K-12 ATPase (Evans, 1969). The optimal ratio for *X. oryzae* is 1.5. It is different from that of the ATPase reported from other bacterial systems, the reason for the difference is unknown. The Mg^{++}-activation curve from *X. oryzae* was similar to those of ATPase from *S. aureus*, *M. lysodeikticus*, *L. arabinosus* and *E. coli* K-12. The curves are not belong to a sigmoidal type curve, therefore, Mg^{++} is not like acting as an effector or a modulator of the enzyme. It may have a similar role to other membrane-bound Mg^{++}-activated ATPase reported.

Optimum pH of cell free ATPases varied with different kind of bacteria. The optimum pH of *Staphylococcus aureus* ATPase is about 6.0; and that of

Streptococcus faecalis ATPase is 8.0. In *E. coli* K-12 the optimum pH for ATPase is very high and with little or no activity at neutral pH. Since ATPase may have conformational change after it was released from membrane, the optimal pH in vitro may not be the same in vivo. In *Xanthomonas oryzae*, ATPase associated with membrane and that released from membrane both have an optimum pH at 7.5.

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水稻白葉枯病病原菌細胞膜上腺嘌呤核苷三磷酸分解酵素的性質

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附着於水稻白葉枯病病原菌細胞膜上的腺嘌呤核苷三磷酸分解酵素需要鎂離子來增加它的活性，在鎂離子與腺嘌呤核苷三磷酸的分子比在 1.5 時酵素的活性最强。另外鎂離子也是酵素附着在細胞膜的一因子，它並且可以增加酵素的隱定性。此酵素可以分解核苷三磷酸及二磷酸，但是對於單磷酸無作用。