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ATPase IN ISOLATED MEMBRANES OF *BACILLUS SUBTILIS*[*]

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SUMMARY

Cytoplasmic membranes from *Bacillus subtilis* ATCC 6633 were prepared by osmotic shock of protoplasts formed by the action of lysozyme. The resulting membrane ghosts were found to possess both Mg^{2+}- and Ca^{2+}-dependent ATP phosphohydrolase (ATPase, EC 3.6.1.3) activities. A maximal activity of 1.67 μmoles of orthophosphate released/min per mg of protein was obtained at pH 8.7 with the Mg^{2+}-ATPase. Maximal activity was observed at a molar Mg^{2+} to ATP ratio of 0.5 and a molar Ca^{2+} to ATP ratio of 0.7 at pH 7.8. Addition of Mg^{2+} and Ca^{2+} together to the assay resulted in an inhibition of both activities. Concentrations of ATP in excess of 2.5 mM inhibited the Mg^{2+}- and Ca^{2+}-dependent enzyme activities, whereas non-enzymatic ATP hydrolysis was inhibited by concentrations of ATP in excess of 5 mM. The 5′-triphosphates of guanosine and uridine were also hydrolyzed by membrane preparations. Na^+ and K^+ each stimulated Mg^{2+}-ATPase activity. Ouabain did not reverse Na^+- or K^+-, or $(Na^+ + K^+)$-mediated enzyme stimulations. The influences of various metabolic inhibitors and other substances on membrane ATPase were studied.

INTRODUCTION

The cationic serum bactericidal agent, β-lysin, is thought to exert its lethal effect in *Bacillus subtilis* at the membrane level[1,2]. One approach to the elucidation of the effects of β-lysin and other cationic bactericidal agents on membranes of this organism is to study their effects on membrane-bound enzyme activities. The ATPase of *B. subtilis* was chosen for this study since ATPases are usually associated with biological membranes. They have been found to be attached to plasma membranes of eucaryotic

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Abbreviations: HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; TAPS, tris-(hydroxymethyl)methylaminopropanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; DCCD, *N*,*N*′-dicyclohexylcarbodiimide; PCMB, *p*-chloromercuribenzoate; DDT, 1,1,1-trichloro-2,2-bis-(*p*-chlorophenyl)ethane; DDE, 1,1-dichloro-2,2-bis-(*p*-chlorophenyl)ethylene.

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cells[3,4] as well as to the membranes of their organelles[5–7] and to the cellular membranes of several procaryotes[8–17].

Since the properties of this enzyme from *B. subtilis* have not been previously reported, the enzyme was characterized as the first step in the proposed studies. The characterization of this ATPase was also thought to be potentially useful since the genetics of *B. subtilis* has been studied extensively. In addition, a study of this enzyme was considered to be of interest since ATPases have been implicated in many cellular functions, including energy generation[7,9,14,18,19], mitochondrial contraction[20], muscular contraction[21], biochemical syntheses, and active transport[4,22–24]. The properties of the membrane ATPase are described in this paper. A manuscript describing the influences of β-lysin and other cationic bactericidal agents on this enzyme is in preparation.

MATERIALS AND METHODS

Materials

Sodium salts of the nucleoside triphosphates and of ADP, ouabain, egg-white lysozyme, antimycin A, quinacrine(atebrin)·HCl, 2,4-dinitrophenol grade II, and oligomycin were purchased from the Sigma Chemical Company, St. Louis, Mo. Tris buffer was obtained from Schwartz Bioresearch, Inc., Orangeburg, N.Y. *N*-2-Hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), tris-(hydroxymethyl)methylaminopropanesulfonic acid (TAPS), 2-(*N*-morpholino)ethanesulfonic acid (MES), *N*,*N'*-dicyclohexylcarbodiimide (DCCD), and *p*-chloromercuribenzoate (PCMB) were obtained from Calbiochem, Los Angeles, Calif. Deoxyribonuclease was purchased from Worthington Biochemical Corp., Freehold, N.J. ELON, a photographic developer, was purchased from Kodak, Rochester, N.Y. Sodium dithionite and pentachlorophenol were supplied by J. T. Baker Chemical Co., Phillipsburg, N.J. 1,1,1-Trichloro-2,2-bis-(*p*-chlorophenyl)ethane (DDT) and 1,1-dichloro-2,2-bis-(*p*-chlorophenyl)-ethylene (DDE) were generously donated by Dr S. A. Peoples of the University of California at Davis. All other chemicals were of reagent grade. Stock solutions of DCCD, pentachlorophenol, DDT, DDE, antimycin A, and oligomycin were prepared in 95% ethanol. Substrates were adjusted to pH 7.2 with 1 M NaOH and kept frozen.

Preparation of cell membranes

B. subtilis was grown to late log phase (12 h) at 37 °C in the liquid medium of Roberts and Johnson[25] modified by the addition of 0.075% yeast extract[2]. The cells were harvested by centrifugation and then washed twice with 50 mM Tris–HCl buffer, pH 7.2, containing 10 mM $MgCl_2$. The washed cells were suspended in a stabilizing medium containing 0.2 M sucrose and 10 mM $MgCl_2$ in 50 mM Tris–HCl buffer, such that the absorbance at 650 nm of a 1:10 dilution of this suspension was 0.6–0.9. Crystalline lysozyme was then added to give a final concentration of 100 μg/ml. During incubation for 35 min at 37 °C, the cells were converted to protoplasts which were harvested by centrifugation. The protoplast pellet was suspended in 50 ml of cold 50 mM Tris–HCl, pH 7.2, containing 0.5 mM $MgCl_2$. Sufficient solid deoxyribonuclease was added to the membrane suspension to bring the concentration to 20 μg/ml and the suspension was incubated for 30 min at 37 °C. The osmotic shock resulted in lysis of the protoplasts with the formation of membrane ghosts which were then separated from the soluble cytoplasmic fraction by centrifugation at $30900 \times g$ for 30 min. The cell membranes were washed twice with 50-ml portions of 50 mM Tris–HCl buffer, pH 7.2, containing 5 mM $MgCl_2$ and were then suspended in this buffer. For experiments involving activation by Ca^{2+}, aliquots of a membrane suspension were washed twice and resuspended in Tris–HCl buffer without added divalent cation.

ATPase assay

The reaction mixtures contained 2.5 mM ATP, 1.25 mM $MgCl_2$ or 1.75 mM $CaCl_2$, 75 mM Tris–HCl buffer, pH 7.8, and approximately 0.25–0.3 mg membrane protein as determined by the method of Lowry *et al.*[26] in a total volume of 2.0 ml and were incubated at 37 °C. After 15–20 min, the reaction was terminated by making the mixture 2.5% in cold $HClO_4$. Approximately 10% of the total substrate was converted to orthophosphate (P_i) during this time. The tubes were centrifuged for 5 min to remove precipitated protein, and up to 1.2-ml aliquots were removed for the analysis of P_i. The liberation of P_i was estimated by the following modification (personal communication from Dr Jack Preiss, Department of Biochemistry and Biophysics, University of California, Davis) of the Fiske–SubbaRow method[27]. To the 1.2-ml aliquots to be analyzed for P_i, 0.2 ml of 2.5 M H_2SO_4, 0.2 ml 2.5% $(NH_4)_6Mo_7O_{24} \cdot 4\ H_2O$ and 0.4 ml 1% ELON in 3% $Na_2S_2O_5$ were added in the order listed. The absorbances of the samples were read in exactly 10 min at 700 nm on a Beckman DB-G spectrophotometer. The analytical values were corrected for the non-enzymatic hydrolysis of substrate and for the contents of extraneous P_i in the enzyme preparation and in the reagents.

Enzymatic activity was proportional to the concentration of membrane protein up to 0.7 mg protein per reaction mixture and proportional with time of incubation at 37 °C up to at least 45 min.

Under the above described conditions of membrane preparation and enzyme assay, the range of specific activities of the membrane-bound, Mg^{2+}-activated ATPase was 50–90 nmoles P_i liberated$\cdot min^{-1} \cdot mg^{-1}$ protein. Approximately 80–90% of the total ATPase activity of the lysed protoplasts was membrane-bound.

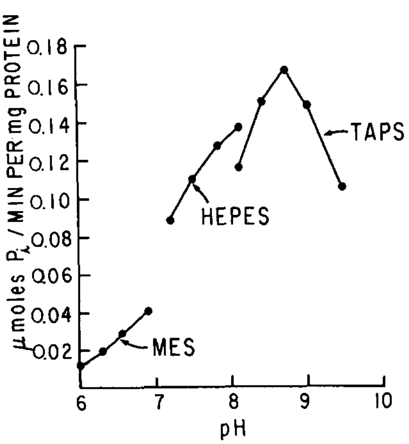
RESULTS

Influence of pH on Mg^{2+}-ATPase activity of membranes

Fig. 1 shows the effect of varying the pH between 6.0 and 6.9 in MES, between 7.2 and 8.1 in HEPES, and between 8.1 and 9.45 in TAPS on the Mg^{2+}-ATPase activity of the membrane preparations. The activity gradually increased until maximal activity at pH 8.7 was reached. Beyond pH 8.7 a sharp drop in enzyme activity occurred.

Metal ion dependency of ATPase activity

Fig. 2 shows that ATPase activity was not present when Mg^{2+} and Ca^{2+} were omitted from the assay. The enzyme was maximally activated by Mg^{2+} at a molar ratio of Mg^{2+}:ATP equal to 0.5; however, only slightly less activation occurred at Mg^{2+}:ATP ratios of 0.3 and 0.6. Ca^{2+} was found to be a better activator of the *B. subtilis* membrane ATPase than Mg^{2+}. The enzyme was maximally activated by Ca^{2+} at a molar ratio of Ca^{2+}:ATP of 0.7.



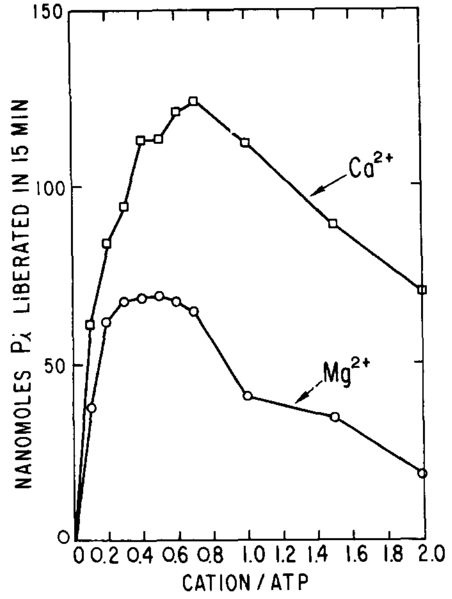


Fig. 1. Influence of pH on the enzymatic hydrolysis of ATP. Buffers were prepared by adjusting each to the desired pH with HCl or NaOH. With the exceptions of variations in buffer and in pH, conditions of incubation and assay were as described in Materials and Methods.

Fig. 2. Divalent cation dependency of membrane ATPase. The reaction mixtures contained 75 mM Tris–HCl buffer (pH 7.8), 2.5 mM Na_2 ATP, and 0–5 mM $MgCl_2$ or $CaCl_2$. Other conditions of incubation and assay were as described in Materials and Methods.

The addition of the maximally activating concentrations of Mg^{2+} and Ca^{2+} together to the assay was observed to partially inhibit enzyme activity as follows: the addition of Ca^{2+} decreased Mg^{2+}-stimulated ATPase to 70% of its original activity, while the addition of Mg^{2+} reduced the Ca^{2+}-stimulated activity to 20% of its original value.

Influence of ATP concentration on hydrolysis of substrate

The experiments described in Fig. 3 show that concentrations of ATP in excess of 2.5 mM inhibited both the Mg^{2+}- and Ca^{2+}-stimulated enzymatic activities, when the divalent cations were kept constant at their maximally activating concentration for 2.5 mM ATP. This is an indication that free ATP appears to be an inhibitor of the substrate, probably a divalent cation–ATP complex.

ATP will spontaneously hydrolyze in the absence of enzyme. The non-enzymatic hydrolysis was also inhibited by excess ATP. The maximal P_i release occurred at 5 mM ATP, whereas the enzymatic ATP hydrolysis had its maximum at 2.5 mM ATP.

Substrate specificity

Other nucleoside phosphates were tested as substrates in place of ATP. It can be seen from Table I that GTP and UTP were split by the membrane preparations at appreciable rates: 235% and 180% of the ATP value, respectively. CTP and ADP

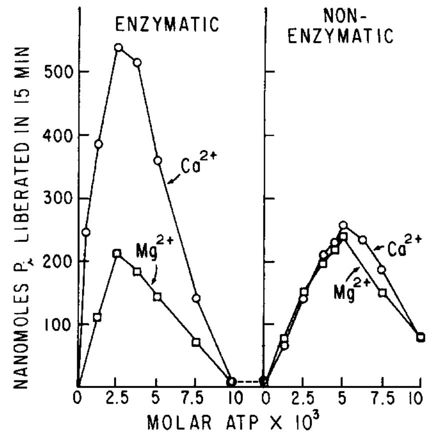


Fig. 3. Influence of varying the concentration of ATP on the enzymatic and non-enzymatic hydrolysis of this compound. The reaction mixtures contained 75 mM Tris–HCl buffer (pH 7.8), 1.25 mM $MgCl_2$ or 1.75 mM $CaCl_2$, 0–10 mM Na_2 ATP, and 0.28 mg membrane protein for enzymatic hydrolysis. No membrane was added in the case of non-enzymatic ATP hydrolysis; the reaction was started by the addition of ATP. Other conditions were as described in Materials and Methods.

TABLE I

SUBSTRATE UTILIZATION BY MEMBRANE PREPARATIONS

Conditions of assay were as described in Materials and Methods except for variations in substrate. Reaction mixtures contained 2.5 mM substrate. Results are expressed as percentages of the rate with ATP.

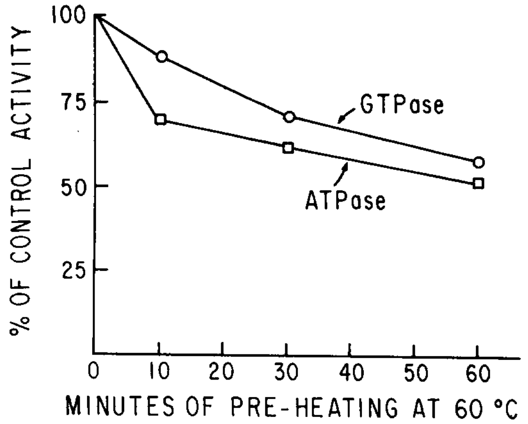
Substrate	*% ATP hydrolysis*
ATP	100
CTP	0
GTP	235
UTP	180
ADP	0
ATP+ADP	0
GTP+ADP	0

were not hydrolyzed. ADP at a concentration equimolar to ATP or GTP inhibited the ATP and GTP hydrolyzing activities of the membranes.

As can be seen in Fig. 4, the hydrolysis of ATP and GTP followed similar kinetics of heat denaturation at 60 °C. After 1 h at 60 °C, about 50% of the ATP hydrolyzing activity and 58% of the GTP hydrolyzing activity remained. This result suggested that both activities reside in the same protein.

Influences of monovalent cations on the membrane-bound Mg^{2+}-ATPase activity

Fig. 5 shows that the monovalent cations Na^+ and K^+ stimulated the Mg^{2+}-dependent membrane-bound ATPase activity. Enzyme saturation by Na^+ occurred



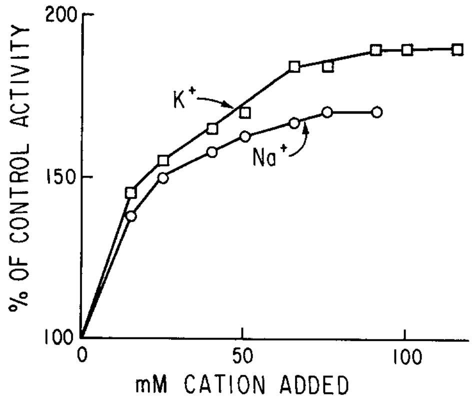


Fig. 4. Heat inactivation of ATP and GTP hydrolyzing activities. 1.6 ml of a membrane suspension was heated to 60 °C. 0.5-ml samples were withdrawn at 10, 30 and 60 min and assayed as described in Materials and Methods for ATP and GTP hydrolyzing activities using 2.5 mM ATP and 2.5 mM GTP, respectively.

Fig. 5. Stimulation of ATPase activity by Na^+ or K^+. NaCl or KCl was added in the concentrations indicated. Other conditions were as described in Materials and Methods.

at a concentration of 75 mM and stimulation amounted to 170% of the activity exhibited in the absence of Na^+ or K^+. Saturation of the ATPase by K^+ occurred at 90 mM KCl; activity was increased to 190% of the basal level at this K^+ concentration. Mixtures of saturating amounts of Na^+ and K^+ showed no additive stimulating effect, whereas mixtures of sub-saturating concentrations were additive. These results suggested that Na^+ and K^+ acted at the same site and in the same manner to stimulate enzyme activity.

To determine if these stimulations were simply the result of increases in ionic strength due to the presence of significant concentrations of the monovalent cations, the enzyme was assayed in the presence of increasing concentrations of Tris–HCl and, separately, of increasing concentrations of HEPES. A progressive decline in enzyme activity was observed as the concentration of each of these buffers was increased. This result indicates that the stimulations by the metal ions were specific and were not due to increases in ionic strength.

Stimulations of enzyme activity due to Na^+, K^+, or $Na^+ + K^+$ were not susceptible to reversal by the cardiac glycoside, ouabain, at concentrations as high as 1 mM.

Influences of various compounds on the Mg^{2+}-dependent membrane-bound ATPase

The influences of various compounds on the hydrolysis of ATP by the Mg^{2+}-dependent membrane ATPase of *B. subtilis* are presented in Table II. The effect of ethanol on membrane Mg^{2+}-ATPase activity was studied since many of the substances investigated in this section were not water soluble. Increasing concentrations of ethanol in the range 0 to 16.5% increasingly activated enzyme activity. Activation reached 450% at 16.5% ethanol. 0.1 mM PCMB, 1 μM DDT, and 1 μM DDE had no effect on the enzyme. 1 mM 2,4-dinitrophenol was slightly inhibitory, leaving 87% of

TABLE II

EFFECTS OF VARIOUS COMPOUNDS ON THE HYDROLYSIS OF ATP

Conditions of assay were as described in Materials and Methods except for the addition of the compounds listed to the assay mixtures.

Compound	*Concentration*	*ATPase activity in percent of control*
Ethanol	16.5%	450
PCMB	0.1 mM	100
DDT*	1 μM	100
DDE*	1 μM	100
2,4-Dinitrophenol	1 mM	87
NaN_3	1 mM	7
DCCD**	50 μM	16
Atebrin	0.5 mM	10
$Na_2S_2O_4$	1 mM	286
Na_2SO_3	1 mM	257
Oligomycin*	62.5 μM	136
Pentachlorophenol*	1 mM	320
Antimycin A*	0.1 mM	205

* Added to assay in an ethanolic (95%) solution. Results were corrected for the effect of ethanol on ATPase activity.

** Added to assay in an ethanolic (95%) solution.

the control activity. 1 mM NaN_3, 50 μM DCCD, and 0.5 mM atebrin markedly inhibited enzyme activity leaving 7, 16, and 10% of the control value, respectively. $Na_2S_2O_4$ and Na_2SO_3 (each 1 mM) increased substrate hydrolysis to 286 and 257% of the control, respectively. Also 62.5 μM oligomycin and 1 mM pentachlorophenol activated the enzyme to 136 and 320%, respectively. A maximal enzyme activation of 205% occurred at 0.1 mM antimycin A. Above this concentration (up to 0.5 mM), activation by the antibiotic progressively decreased, but the level of activity did not fall below that of the control.

Stability of ATPase preparations

Membrane-bound Mg^{2+}- and Ca^{2+}-ATPase activities were stable at 4 °C for at least 45 days. Freezing at about −15 °C followed by thawing at 25 °C abolished the membrane-bound activity.

DISCUSSION

The ATPase of *B. subtilis* has many properties in common with ATPases from other sources, particularly those from various *Bacillus megaterium* strains[12,16,29].

The pH optimum of 8.7 for the *B. subtilis* ATPase is in agreement with the values obtained by various workers for the Mg^{2+}-ATPases in different organisms.[5,10,28]. In contrast, an optimum of pH 5.9 to 6.1 was reported for *Staphylococcus aureus*[11].

As is the case with ATPases of higher organisms[4,5,7] and other microorganisms[8,10,16,29,30], the membrane ATPase of *B. subtilis* was dependent for its activity on the presence of a divalent cation. The inhibition of enzyme activity by concentrations of ATP in excess of that giving an optimal divalent cation: ATP ratio has also been reported for membranes of *B. megaterium*[29] and *Bacillus stearothermophilus*[30]; the optimal Mg^{2+}: ATP ratios for the enzymes of these *Bacillus* species were similar to that described here for *B. subtilis*. Optimal Mg^{2+}: ATP ratios of 0.5 were also demonstrated for ATPases from spinach chloroplasts[31] and rat liver mitochondria[32]. Additional organisms have been reported to require other specific ratios of Mg^{2+}: ATP for optimal activity[5,8]. These results suggest that the actual substrate giving maximal enzyme activity for each system is a specific divalent cation–ATP complex and that reduction of divalent cation mediated stimulation of ATP hydrolysis by excess ATP is perhaps due to competition between the divalent cation–ATP complex and free ATP for the active site of the enzyme. It is not surprising that the non-enzymatic hydrolysis of substrate also required a specific divalent cation:ATP ratio for optimal P_i release which differed from that optimal for enzymatic hydrolysis since the thermodynamic potential of the ATP molecule in its equilibrium hydrolysis is believed to change as a function of concentrations of cations such as Mg^{2+} or Ca^{2+} (ref. 33).

Inhibition of divalent cation dependent ATPase activities by the simultaneous presence of Mg^{2+} and Ca^{2+}, as reported here in the case of *B. subtilis*, has also been observed with ATPases of *B. megaterium*[12,16], *Micrococcus lysodeikticus*[15], *Streptococcus faecalis*[34] and beef heart mitochondria[5]. In agreement with the present findings for *B. subtilis*, other workers have found that bacterial and mitochondrial ATPases utilized nucleoside 5′-triphosphates other than ATP and that these activities were not separable[5,12,16,28].

ATPases from various sources are thought to function in Na^+, K^+ transport across cell membranes[4,22,23]. A similar function can be presumed for the ATPase of *B. subtilis* since this enzyme was affected by these ions. In agreement with the results of Abrams[28] for *S. faecalis*, this enzyme exhibited no discrimination between Na^+ and K^+. In contrast to the animal ATPases, the enzyme of *B. subtilis* did not appear to be specifically stimulated by mixtures of Na^+ and K^+. This result corresponds with those reported for other bacteria[11,28]. Some bacterial enzymes, including those of two *B. megaterium* strains, are inhibited by Na^+ or K^+ (refs 10, 12, 35, 36). It therefore appears that monovalent cations can have quite different effects on the ATPase activities of various bacteria. Also, in contrast to animal ATPases, the Na^+ and K^+ stimulations of the *B. subtilis* ATPase were not reversed by the cardiac glycoside, ouabain. A similar result has been reported for the membrane ATPase of *Staphylococcus aureus*[11].

DCCD has been reported to inhibit mitochondrial ATPase activity[37] and to bind covalently to mitochondrial fractions[38]. The membrane ATPase of *S. faecalis* has been shown to be inhibited by DCCD[23]. Evans[39] found that the ATPase of *Escherichia coli* was also sensitive to DCCD. The stimulation of ATPase activities by low concentrations of pentachlorophenol has been found in mitochondria[19,40,41], myosin[41], *E. coli*[10], and *B. megaterium*[12] as well as in *B. subtilis*. The sulfhydral inhibitor PCMB was found by Greenawalt and coworkers[36] to inhibit the membrane-bound Mg^{2+}-dependent ATPase of *B. megaterium*, strain M, to 64% of the control at 0.1 mM PCMB. However, Ishida and Mizushima[12], working with this enzyme from *B. megaterium*, strain NRLL B939, at the same concentration of PCMB found no inhibition. No effect of PCMB on enzyme activity in *B. subtilis* was observed, indicating that the active site of this enzyme does not involve a thiol group.

The reducing agent sodium dithionite has been reported to stimulate ATPases from various biological sources[9,12,31,36]. Ishida and Mizushima[12] reported that the stimulation of the membrane-bound and solubilized ATPases of *B. megaterium* by dithionite was due, not to its reducing property, but to an unexplained effect of sulfite, one of the breakdown products of dithionite in aqueous solution. A similar situation appears to exist for the membrane ATPase of *B. subtilis*, since equimolar concentrations of dithionite and sulfite increased enzyme activity to the same extent.

Oligomycin has been reported to inhibit respiration and oxidative phosphorylation in many eucaryotic organisms and to inhibit the membrane-bound ATPases of mitochondria[42]. Consistent with the finding that oligomycin has no antibacterial activity[43] are the observations that the ATPases of *B. megaterium*[12,36] and *B. subtilis* were not inhibited by oligomycin. In fact, this antibiotic stimulated the ATPase of *B. subtilis* somewhat.

In addition to inhibiting the ATPase of *B. subtilis*, the respiratory poison, sodium azide, has been reported to inhibit ATPases from diverse biological sources[5,10,12,15,31]. Atebrin, an oxidative and photo-phosphorylation inhibitor, inhibited ATPase activities from *B. subtilis*, *S. aureus*[11], *B. megaterium*[36] and chloroplasts[31]. The effects of DDT and its noninsecticidal analogue DDE on the ATPase of *B. subtilis* were tested since two ATPases from rat brain nerve endings were inhibited by these compounds[44]. The enzyme from membranes of *B. subtilis* was insensitive to both DDT and DDE at $1 \cdot 10^{-6}$ M.

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