Adenosine Triphosphatase in Isolated Membranes of Staphylococcus aureus

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The preparation of cytoplasmic membranes from suspensions of Staphylococcus aureus lysed by an enzyme recently isolated in these laboratories is described. These membranes contained: protein, 34.4%; ribonucleic acid, 6.6%; lipids, 34.5%; and total phosphorus, 1.4%. Such membranes exhibited adenosine 5'-triphosphatase (E.C. 3.6.1.3) activity, liberating orthophosphate at an initial rate of 0.53 µmole per min per mg of protein under optimal conditions. The enzyme was Mg++-dependent and K+- or Na+-stimulated. Maximal activity was observed with a molar adenosine 5'-triphosphate (ATP) to Mg++ ratio of 1. One mole of orthophosphate was liberated per mole of ATP; the other product of digestion was adenosine 5'-diphosphate. Inorganic pyrophosphate and the 5'-triphosphates of guanosine, uridine, and cytidine were also attacked by membrane preparations, but more slowly than ATP. Ouabain, p-chloromercuribenzoate, and 2,4-dinitrophenol did not alter adenosine triphosphatase activity, whereas both Atebrine and chlorpromazine were inhibitory.

During a study (6, 7) of penicillinase transport in Staphylococcus aureus (8325; α I-, p+), it became of interest to determine whether adenosine 5'-triphosphatase (ATPase) activity was associated with the cytoplasmic membrane of this organism, as has been found with several other species (2, 11, 17, 22). The preparation of cytoplasmic membranes from S. aureus by means of gentle techniques has, in the past, been difficult, as this organism is resistant to the action of lysozyme. The autolytic technique of Mitchell and Moyle (19) is time-consuming and was not suitable for our purpose. Recently, Coles and Gilbo (5) observed rapid lysis of S. aureus by culture supernatant fluids from a species of Aeromonas. Using the partly purified lytic principle from such culture fluids, we prepared cell membranes from S. aureus. The present communication describes the isolation of the membranes and some of the properties of an ATPase associated with them.

Materials and Methods

Preparation of membranes. The strain of S. aureus used and the method of cultivation were the same as described previously (7). The enzyme preparation used for the lysis of S. aureus was purified to the stage of elution from AG-50W resin (A. J. Broad, C. M. Gilbo, and N. W. Coles, Proc. Intern. Congr. Biochem. Tokyo, 7th, 1967). The cells were washed in glycine (0.02 M final concentration, adjusted to pH 9.0 with sodium hydroxide) and suspended in this buffer to a final absorbance of 0.6 at 660 mµ in a Bausch and Lomb Spectronic-20 colorimeter. Sufficient units of lytic enzyme (5) were added for complete lysis (absorbance reduced to <0.02 at 660 mµ) to occur within 30 min at 28 C. This rather dilute lysate was centrifuged at 5,000 × g for 10 min to ensure that no intact organisms remained. The supernatant fluid was centrifuged for 30 min at 20,000 × g, and the pellets from this centrifugation were pooled and washed three times with 0.01 M tris-(hydroxymethyl)aminomethane (Tris) buffer which had been adjusted to pH 7.6 with HCl and contained 1.0 mM Mg++. Centrifugations between washes were for 15 min at 45,000 × g. The final pellet was suspended in 0.01 M Tris-acetate buffer (pH 6.0) to a final concentration of 0.7 to 1.0 mg of protein per ml.

Protein was determined by the method of Lowry et al. (14) with the use of a bovine serum albumin standard, ribonucleic acid (RNA) was determined by the orcinol method (16), and lipids were determined as described by Folch, Lees, and Sloane Stanley (9). Inorganic phosphate (Pi) was assayed by a modification of the method of Martin and Doty (12). For total phosphorus assays, membranes were hydrolyzed by the method of Bartlett (3). For chemical analysis, membranes were dialyzed free of buffer, dried, weighed, and dispersed in distilled water.

Assays of enzymatic activity. Standard incubation mixtures contained 100 mM KCl, 2 mM MgCl2, 2 mM Tris adenosine 5'-triphosphate (ATP) [prepared according to Epstein and Whitlam (8)], and 50 mM Tris-acetate buffer (pH 6.0). For each assay, the final volume of 1.0 ml contained membrane preparation equivalent to 0.07 to 0.1 mg of protein (the amount
was constant for each experiment). Incubation was at 37 C for 30 min. The reaction was stopped by the addition of 0.5 ml of 1.5 M perchloric acid, and the tubes were centrifuged at 0 C to sediment protein. P$_i$ was assayed in 1.0-ml samples, and values were corrected for controls (enzyme preparation added after perchloric acid).

Ascending paper chromatography was carried out in the isoamyl alcohol-tetrahydrofurfuryl alcohol-0.08 M potassium citrate, pH 7.92 (1:1:1), solvent of Carpenter (4), with the substitution of sodium citrate for potassium citrate. Although very satisfactory for our purpose, the solvent used gave R$_b$ values quite different from those listed by Carpenter (4). Incubation mixtures for chromatography were freeze-dried and resuspended in the above sodium citrate buffer to one-tenth the original volume.

RESULTS

Cytoplasmic membranes. The yield of washed membranes obtained from the strain of S. aureus we used averaged 5 to 6% of the dry weight of cells. This yield was low compared with that reported for a different strain of S. aureus (18) and for other gram-positive organisms (20). When this study was undertaken, any possible effect of the lytic factor on membrane structures was not known. To determine whether such an effect existed, membranes prepared from mechanically disrupted cells were treated for 30 min with five times the enzyme concentration used for the lysis of cells. Membranes thus treated were isolated in the usual way and assayed for total protein and ATPase. Neither of these components was diminished by treatment with the lytic enzyme. A further enzyme activity bound to membranes of this strain of S. aureus, penicillinase (unpublished data), was also fully recovered on the membranes after the above treatment. Membranes contained: protein, 34.4%; ribonucleic acid, 6.6%; lipids, 34.5%; and total phosphorus, 1.4%.

Influence of pH on enzymatic cleavage of ATP by membrane preparations. Figure 1 shows the effect of varying the pH between 5.0 and 10.0 on the ATP-cleaving activity of membrane preparations. Enzyme activity increased sharply with increasing pH until the optimal range of 5.9 to 6.1 was reached. With higher pH values, there was a more gradual decrease in activity.

Effect of Mg$^{++}$ and Ca$^{++}$ on the hydrolysis of ATP by membrane preparations. As in higher organisms and in ATPases of other microorganisms (2, 11, 17, 22), the membrane ATPase of S. aureus was dependent for its activity on the presence of Mg$^{++}$. Figure 2 shows that ATPase activity was absent in the absence of Mg$^{++}$ or Ca$^{++}$. A sharp rise to maximal activity was observed on the addition of Mg$^{++}$. The increase ceased when the molar ratio of ATP to Mg$^{++}$ was 1. Ca$^{++}$ also activated the enzyme, but was much less effective than Mg$^{++}$.

Influence of other cations on ATPase activity. Although ATPase activity was present in buffered medium containing only Mg$^{++}$, ATP, and enzyme...
preparation, the addition of K+ or Na+ caused considerable stimulation. Up to a concentration of 30 mM, both ions had the same stimulating effect. Maximal stimulation by Na+ was obtained at a concentration of 60 mM and amounted to an increase of 65% over the activity exhibited in the absence of Na+ or K+. K+, at a concentration of 100 mM, increased this basal activity by 110% (Fig. 3). Mixtures of Na+ and K+ showed no additive stimulating effect.

End products of enzymatic hydrolysis of ATP. Paper chromatography of the incubation mixture for the detection of purine derivatives after 0, 10, 30, and 60 min of incubation showed progressively more adenosine 5'-diphosphate (ADP) and less unchanged ATP. No other adenine derivatives could be demonstrated. Quantitative studies indicated that no more than 1 mole of P1 was liberated per mole of ATP (Fig. 4). The initial reaction rate corresponded to the release of 0.53 μmole of P1 per min per mg of protein.

Specificity of membrane preparations. Other nucleoside 5'-triphosphates were tested as substrates of membrane ATPase. After 30 min of incubation under conditions optimal for ATPase activity, their hydrolysis, compared with that of ATP, was as follows: guanosine 5'-triphosphate (GTP), 70%; uridine 5'-triphosphate (UTP), 7%; and cytidine 5'-triphosphate (CTP), 4%. Inorganic pyrophosphate (PPi) was also attacked under these conditions, its hydrolysis being 15% of that of ATP.

Stability of ATPase preparations. Membranes stored at pH 7.6 at temperatures between 0 and 37°C had lost no activity after 19 hr. Inactivation during storage for longer periods in distilled water or buffer at 4°C varied with different preparations. Some membrane preparations exhibited diminished ATPase activity on storage under these conditions for periods up to 8 days, whereas others had lost 80% of their original activity after 7 days. Repeated freezing and thawing of stable preparations did not result in loss of activity.

Effect of inhibitors on ATPase activity. The inclusion in the incubation medium of ouabain (1.0 mM) or p-chloromercuribenzoate (1.0 mM) did not diminish ATPase activity. Atebrine (10 mM) and chlorpromazine (0.4 mM) inhibited the enzyme 70 and 64%, respectively. Enzyme activity was not affected by 2,4-dinitrophenol (0.1 mM).

Attempts to solubilize membrane ATPase. Attempts to release ATPase in soluble form from membranes of S. aureus by repeated washing with dilute buffer in the absence of multivalent cations, a procedure used by Abrams (1) to solubilize Streptococcus faecalis membrane ATPase, were not successful. A small percentage (5%) of activity appeared in the supernatant fluid of the first two washes, but this dropped to zero in subsequent washings.

**DISCUSSION**

The ATPase associated with cytoplasmic membranes of S. aureus has properties similar to those
of ATPases from membranes of other microorganisms, but does not correspond in all its properties to any one of those described in the literature. Table 1 lists some of the properties of ATPases found associated with membranous structures of several microorganisms, and compares them with those of the ATPase described in this paper.

The staphylococcal membrane enzyme appears to be a true ATPase (E.C. 3.6.1.3) which splits only the terminal phosphate from ATP. The dependence of activity on the presence of Mg\(^{++}\) and the optimal ratio of ATP to Mg\(^{++}\) of 1 suggest that the substrate is Mg ATP.

In contrast to the membrane ATPase of *Bacillus megaterium* (10), the enzyme described here is not inhibited by p-chloromercurbicyanate and, therefore, does not appear to depend on -SH groups for activity. Löw (13) found that both Atebrine and chlorpromazine inhibit the Mg\(^{++}\)-activated rat liver mitochondrial ATPase, and suggested that they inhibit flavoproteins which take part in the ATPase reaction. A similar mechanism may apply to staphylococcal membrane ATPase, which is also inhibited by both Atebrine and chlorpromazine.

K\(^{+}\), and to a less extent Na\(^{+}\), have a strongly stimulating effect on the Mg\(^{++}\)-activated staphylococcal enzyme, but in contrast to the Na\(^{+}\) and K\(^{+}\)-activated ATPase of mammalian tissues and erythrocytes (23, 24), this stimulation is not susceptible to inhibition by ouabain. Insensitivity to ouabain inhibition has also been shown for the Na\(^{+}\)- or K\(^{+}\)-stimulated ATPase of *Vibrio parahaemolyticus* membranes (11). In view of the stimulating effect of Na\(^{+}\) and K\(^{+}\) on the staphylococcal membrane ATPase, the possibility of its participation in active ion transport must be considered.

The data presented do not allow further speculations on the function of ATPase in the membranes of intact staphylococci, but the presence of this enzyme within the permeability barrier of the cell is consistent with the postulated role of ATP in processes of active transport across the cell membrane (21).

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**LITERATURE CITED**


3. Bartlett, G. R. 1959. Phosphorus assay in...