Reversible Attachment of Adenosine Triphosphatase to Streptococcal Membranes and the Effect of Magnesium Ions*

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ABSTRACT: When adenosine triphosphatase (ATPase) released from *Streptococcus fecalis* membranes was added back to the depleted membranes, the enzyme and membranes recombined. The amount of enzyme that attached to a given amount of depleted membranes depended on the concentration of free ATPase and approached a maximum in the presence of a large excess of free ATPase. The addition of Mg^{2+} ions acted to increase the strength of attachment of the enzyme to the membranes but did not increase the total number of binding sites. The amounts of bound ATPase in the fully reconstituted and in the native ATPase–membrane complex were approximately the same and accounted for about 2% of the total membrane protein. By means of zone sedimentation and zone electrophoresis experiments it was shown that the reconstituted ATPase–membrane complex dissociated in the absence of free ATPase unless Mg^{2+} was present.

The ATPase did not combine significantly with ribosomes at high Mg^{2+} concentrations. We draw the following conclusions from our findings. (1) *S. fecalis* membranes contain a limited number of specific binding sites which can attach ATPase reversibly. (2) There are at least two types of interaction between the enzyme and its membrane binding site one of which involves Mg^{2+}. (3) Mg^{2+} provides additional linkages between the enzyme and membrane binding site so that the complex remains stable in the absence of free ATPase.

Bacterial plasma membranes apparently contain a wide variety of proteins. In addition to the many enzymes, they include the specific carriers for transport (Fox and Kennedy, 1965) and the so-called structural proteins (Razin *et al.*, 1965; Salton and Netschey, 1965). In general, the nature of the interactions which maintain the membrane proteins, particularly enzymes, in a bound state is not well understood. Green and his collaborators (Green and Perdue, 1966) have emphasized the important role of hydrophobic forces for the stability of mitochondrial membranes. Razin *et al.* (1965) has concluded that both hydrophobic groups and Mg^{2+} ions are involved in reconstituting the membranes of *Mycoplasma* bacteria from subunits. Previous studies in our laboratory (Abrams *et al.*, 1960, 1965, 1967) indicated that multivalent cations are involved in maintaining the ATPase[1]–membrane complex of *Streptococcus fecalis*. In our earlier investigations we found that ATPase is firmly bound to the membrane ghosts derived by osmotic shock or by metabolic lysis of protoplasts in the presence of Mg^{2+}. The enzyme could be subsequently detached from the membranes more or less selectively in an unexpected way. When the membrane ghosts were washed repeatedly with Tris buffer without Mg^{2+}, the enzyme remained attached even after six or seven washes, but in the next two washes there was a sudden almost quantitative release of the enzyme in true soluble form (Abrams, 1965). Most significant is the fact that the detachment of the enzyme was completely prevented if Mg^{2+} or other multivalent cations were introduced into the wash fluid. This curious delayed "all-or-none" release phenomenon has proved to be remarkably reproducible and very useful as a first step in the purification and characterization of the enzyme (Abrams and Baron, 1967). Another useful feature of this method for releasing the ATPase is that it is far less likely to destroy native membrane structures than the commonly used procedures for isolating membrane components such as the use of detergents and sonication. The mechanism of the delayed all-or-none release is not yet clear. It would appear that during the repeated washing with Tris buffer there is a cumulative weakening of the forces holding the ATPase to other membrane components which leads eventually to an abrupt release. There is evidence that other membrane proteins are also released (Abrams, 1965; Abrams and Baron, 1967). The weakening may be due at least partially to a gradual removal of multivalent cations, probably Mg^{2+}, to below a critical number necessary to hold an ATPase molecule to a membrane site.

After solubilizing the ATPase, we were able to isolate it as an apparently homogeneous protein and elucidate some of its physical and enzymatic properties (Abrams and Baron, 1967). The enzyme is a highly acidic protein with a molecular weight of approximately 350,000

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[1] Abbreviations used: ATPase, adenosine triphosphatase; ADP, adenosine diphosphate.

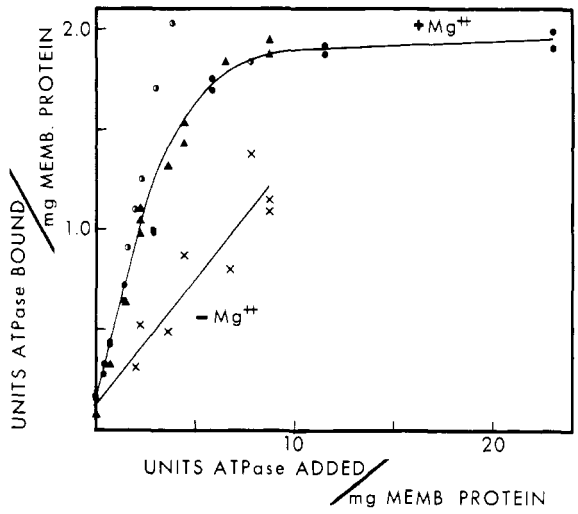

FIGURE 1: Binding *vs.* ATPase concentration. Depleted membranes and ATPase were incubated in 0.2 ml for 10–15 min at 0° in the presence of 10 mM Mg^{2+} and buffer at the pH shown (pH 7.5, 0.1 M Tris-Cl and pH 8.5, 0.0075 M Tris and 0.035 M glycine). The mixtures were then diluted to 1.0 ml with 10 mM Mg^{2+} and buffer and centrifuged for 1 hr at 55,000*g*, and the supernatants were withdrawn. Each pellet was suspended in 1.0 ml of 0.1 M Tris-Cl, pH 7.5, and 0.1- or 0.2-ml aliquots were assayed for ATPase activity. The mixtures incubated without Mg^{2+} were handled in exactly the same manner. Preparation I, pH 7. (◑—◑); preparation II, pH 7.5 (▲—▲); preparation III, pH 7.5 and 8.5 (●—●); preparations I and II, pH 7.5, no magnesium (×—×).

and it is composed of nonidentical subunits. It has an absolute requirement for Mg^{2+} (or Mn^{2+}) for activity. It is strongly inhibited by ADP. The enzyme appears to be specific for purine nucleoside triphosphates (Abrams and Baron, 1967) including deoxyadenosine triphosphate (unpublished results).

The availability of the solubilized membrane ATPase together with the corresponding depleted membranes in a relatively undamaged state made it possible to study the interactions between them by means of recombination experiments. In this paper we will present experiments indicating that reconstitution of the native ATPase–membrane complex can be achieved. We have examined particularly the effect of Mg^{2+} on the recombination process and its reversibility. Our results show that recombination between free ATPase and depleted membranes is reversible and does not require Mg^{2+}; however the presence of Mg^{2+} imparts additional stability to the reconstituted complex and prevents its dissociation.

Methods and Materials

Preparation of Solubilized ATPase and "Depleted Membranes." The procedures for preparing solubilized ATPase and the corresponding depleted membranes from *S. fecalis* membrane ghosts were adapted from those described previously (Abrams *et al.*, 1965, 1967). For a typical preparation used in the present experiments, cells (*S. fecalis* ATCC 9790) were grown at 38° in 1200 ml of a tryptone–yeast extract–potassium phosphate medium (Abrams, 1958) and harvested by centrifugation shortly after reaching the stationary phase of growth. After three washings with cold water, the cells were converted to protoplasts by digestion with lysozyme (120 μg/ml) for 1 hr at 38° in 180 ml of 0.001 M $MgCl_2$ and 0.4 M glycylglycine (pH 7.2) which acts as an osmotic stabilizer (Abrams and McNamara, 1962). To lyse the protoplasts they were first sedimented at 20,000*g* for 10 min and then resuspended with the aid of a syringe in 75 ml of an ice-cold solution containing 0.001 M $MgCl_2$ and 300 μg of DNase. The consequent disruption of the protoplasts due to osmotic shock is virtually complete as judged by the uniformly empty appearance of the resulting membrane ghosts when they are examined by phase microscopy and by electron microscopy (Abrams *et al.*, 1959, 1964). After two washings with 0.001 M Mg^{2+}, the membrane ghosts contained 85% of the total ATPase activity of the protoplast. They contained no tRNA or acetate activating enzyme both of which were found entirely in the cytoplasmic fraction.

The ATPase was eventually released by further repeated washings of the membrane ghosts without Mg^{2+} according to a schedule slightly modified from that described previously (Abrams, 1965). The procedure was carried out at 5° by repeated centrifugation at 150,000*g* and resuspension as follows: twice with 30 ml of 2 M LiCl plus 0.25 M Tris-Cl (pH 7.5), twice with 20 ml of 0.033 M Tris-Cl (pH 7.5), and three to five times with 0.001 M Tris-Cl (pH 7.5). About 70% of the enzyme appears in the supernatant fluid of the seventh to ninth wash. In order to obtain the enzyme in a concentrated form suitable for recombination experiments, we precipitated the solubilized enzyme with $(NH_4)_2SO_4$, between 30 and 80% saturation, at 0° and pH 7.5; the precipitate was then dissolved in a small volume of Tris buffer, 0.02 M (pH 7.5), and it was finally dialyzed to yield a solution containing about 20 units of ATPase/ml. The corresponding membrane residue remaining after release of the enzyme was used in the reconstitution experiments. This residue will be referred to as depleted membranes. It contains a small amount of bound ATPase activity.

Other Methods. For zone electrophoresis we used water-cooled slabs of 5% polyacrylamide gel in Tris-glycine buffer (pH 8.5) in the apparatus manufactured by E. C. Corp., Philadelphia, Pa. (Raymond, 1962). Zones of ATPase activity in the gel were visualized by direct application of ATP followed by reagents for detecting P_i according to the procedure of Abrams and Baron (1967).

Assays of ATPase activity were performed as described previously (Abrams, 1965). A unit of activity is that amount of enzyme which liberates 1 μmole

of P_i/min at pH 7.5 and 38°. Protein was determined by the method of Lowry *et al.* (1951).

Experimental and Results

The Effect of Free ATPase and Mg^{2+} Ion Concentrations on Binding of Enzyme to Depleted Membranes. To determine the effect of free ATPase concentration on binding, depleted membranes were mixed with varying concentrations of the enzyme in the presence of 0.01 M Mg^{2+} ions and in the absence of Mg^{2+} ions. The ATPase activity that became associated with the membranes was then determined by centrifuging the mixture and assaying the pellets. It was not found necessary to wash the pellets since they were very small, about 0.1 mg of protein, so that the amount of free ATPase in the adhering supernatant was insignificant. In control tests, centrifugation of a mixture of free ATPase and Mg^{2+} without depleted membranes yielded either no sedimentable ATPase or very small amounts due to the tendency of the enzyme to aggregate. In the latter case corrections were applied to the amount of enzyme bound.

We carried out these binding experiments with three different preparations of depleted membranes and the corresponding semipurified solubilized ATPase. With one preparation we tested recombination both at pH 7.5 and 8.5. The results obtained with all three preparations are plotted together in Figure 1 as units of ATPase *bound* per milligram of membrane protein *vs.* units of ATPase *added* per milligram of membrane protein. As can be seen in Figure 1 (upper curve) the extent of binding, with Mg^{2+} held constant at 0.01 M, followed a saturation type curve, which was essentially the same for all three preparations; the amount of enzyme bound increased in an almost linear fashion with increasing levels of added enzyme until a maximum was reached when the amount of free ATPase was about five times in excess over the amount of enzyme bound. The maximum amount of recombination could not be increased by doubling the level of Mg^{2+} from 0.01 to 0.02 M. There was no significant difference in binding at pH 7.5 and 8.5. The specific activity of the maximally reconstituted complexes was about 2 units/mg of membrane protein. This value is not greatly different from the specific activities of the original native complexes which were 2.45, 2.62, and 1.70 units per mg of membrane proteins for preparations I, II, and III, respectively, just prior to the release of the enzyme. Since the specific activity of the pure enzyme is about 60 units/mg of protein (Abrams and Baron, 1967) it can be calculated that the attached ATPase accounts for about 2% of the total membrane protein when the membrane is fully saturated with the enzyme. The reasonably close correspondence between the maximum specific activities of the artificial complex and the specific activities of the original native complexes provides strong evidence that we have restored the enzyme molecules to their original sites in the membrane.

We obtained further evidence for the specificity of the membrane binding sites in an experiment showing that the original undepleted membranes failed to take up ATPase on addition of a large excess of the solubilized enzyme (Table I). The free ATPase that was added back to the undepleted membranes was extracted from a portion of the same batch of undepleted membranes. The corresponding depleted membranes, on the other hand, did take up the added free ATPase as expected and the specific enzyme activity of the membranes was restored to its original value (Table I). The inability of *undepleted* membranes to attach more ATPase clearly indicates that the binding sites on the native membranes were already occupied by enzyme molecules and no other sites were available; furthermore, after detachment of the enzyme the emptied sites were apparently reoccupied by the added free ATPase.

TABLE I: Specificity of Membrane Binding Sites for ATPase.

Soluble ATPase Added (units)[a]	ATPase Bound to Membranes[b]	
	Undepleted Membranes	Depleted Membranes[a]
None	1.26	0.16
1.6	1.23	1.23

[a] The solubilized ATPase and the corresponding depleted membranes were prepared from a portion of the undepleted membranes. After solubilization, the enzyme was precipitated with 80% $(NH_4)_2SO_4$ and then redissolved in a small volume of Tris buffer (pH 7.5); insoluble material was removed from the soluble ATPase by centrifugation for 0.5 hr at 150,000*g*. [b] The test for binding was carried out in 0.01 M Mg^{2+} as described in Figure 1; 0.145 mg of undepleted and 0.13 mg of depleted membrane protein were used. Units per milligram of membrane protein.

When Mg^{2+} ions were omitted from mixtures of depleted membranes and *low* concentrations of ATPase, the amount of enzyme bound was still considerable but less than that observed with Mg^{2+} (lower curve in Figure 1). This binding curve in the absence of Mg^{2+} appeared to be approaching the same maximum as that observed with Mg^{2+} (upper curve in Figure 1). It may be concluded, therefore, that Mg^{2+} acts to enhance the strength of binding of the enzyme to the depleted membrane without increasing the total number of binding sites on the membrane.

A study of the effect of varying the Mg^{2+} levels on reconstitution of the ATPase–membrane complex at a constant nonsaturating level of free ATPase is presented in Figure 2. There was considerable binding with no Mg^{2+} added, as mentioned earlier, and the binding was progressively enhanced with increasing Mg^{2+} concentrations until a maximal binding was achieved. With the particular level of ATPase chosen

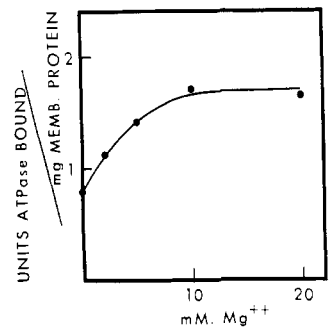

FIGURE 2: Binding *vs.* Mg^{2+} concentration. Depleted membranes, 0.11 mg of protein, and ATPase (0.76 unit) were incubated in 0.2 ml for 10–15 min at 0° with 0, 2, 5, 10, and 20 mM Mg^{2+} and 0.1 M Tris-Cl (pH 7.5). The mixtures were then diluted to 1.0 ml with pH 7.5 buffer and the appropriate concentration of Mg^{2+} and centrifuged for 1 hr at 55,000*g*, and the supernatants were withdrawn. Each pellet was suspended in 1.0 ml of pH 7.5 buffer and duplicate 0.2-ml aliquots were assayed for ATPase activity. The duplication agreed to within ±5%.

for this experiment, maximal binding of enzyme was attained at about 0.01 M Mg^{2+} and 50% of maximal binding was obtained when no Mg^{2+} was added to the system. The binding without added Mg^{2+} could conceivably be due to residual Mg^{2+} remaining in the depleted membranes. However, pretreatment of the membranes with EDTA did not affect their binding capacity.

Stabilization of the ATPase–Membrane Complex by Mg^{2+} during Zone Electrophoresis. Since Mg^{2+} was not required for binding but enhanced the affinity of the ATPase for the membranes (Figures 1 and 2), we postulated that Mg^{2+} probably formed an additional link joining the enzyme to the specific membrane binding site to produce a ternary complex. In this postulated ternary complex all three components are in contact with each other; in the simplest form it may be represented diagrammatically as

$$Mg^{2+}$$
$$/ \quad \backslash$$
ATPase–membrane

This view is consistent with our observation in an earlier study that the addition of Mg^{2+} prevents release of the enzyme from the native complex (Abrams, 1965). It is to be expected that the presumed ATPase–membrane–Mg^{2+} complex once it is formed should not readily dissociate in a medium containing Mg^{2+} without free enzyme, particularly if there are many Mg^{2+} atoms per mole of complex. This prediction was born out by the zone electrophoresis experiment shown in Figure 3. We had shown previously (Abrams and Baron, 1967) that free ATPase migrates electrophoretically in polyacrylamide gel as a well-defined zone whose position is readily located by direct visualization of enzyme activity on the gel. On the other hand, membrane-bound ATPase cannot be expected to migrate into the gel, due to the large size of the particles. Therefore, zone electrophoresis of the reconstituted complex in polyacrylamide gels with and without Mg^{2+} ions in the gel provides a means of demonstrating that the mass action effect of free magnesium alone is sufficient to maintain the integrity of the reconstituted complex. To perform this experiment we first prepared the reconstituted complex in the presence of Mg^{2+} as described in the previous section and then removed it from excess free ATPase and Mg^{2+} by sedimentation. The pellet was suspended in a small volume of Tris-glycine buffer containing Mg^{2+} and samples were electrophoresed in a gel containing 10 mM Mg^{2+} (gel A) and also in a gel not containing Mg^{2+} (gel B). The buffer compartments used for gel A also contained Mg^{2+}. As controls, we ran free ATPase plus Mg^{2+} and depleted membranes plus Mg^{2+} on both types of gels. It can be seen that in the gel without Mg^{2+} (gel B) the ATPase in the complex was released since the enzyme migrated to the position corresponding to free ATPase; on the other hand, in the gel with Mg^{2+} (gel A) the ATPase in the complex was not released but remained at the origin. It should be noted that *free* ATPase migrates into the gel whether or not Mg^{2+} is present.

Stabilization of the Reconstituted ATPase–Membrane Complex by Mg^{2+} during Zone Sedimentation. Zone sedimentation experiments provided more evidence that Mg^{2+} forms additional bonds between the ATPase and the membrane in the reconstituted complex. We mixed depleted membranes, Mg^{2+}, and excess ATPase under conditions shown previously to reconstitute the complex. One aliquot of the mixture was sedimented as a zone through a sucrose gradient containing Mg^{2+}; another aliquot was sedimented in a similar fashion but with no Mg^{2+} in the gradient. A small volume of a very dense sucrose solution was placed at the bottom of each tube providing a cushion to catch the membranes as a sharp zone a short distance from the bottom. Free ATPase, present in great excess, remains near the top of the tube at the centrifugal speeds that were used (Abrams and Baron, 1967). The analysis of the distribution of enzyme activity in the two gradients is shown in Figure 4a,b. It can be seen that the enzyme did not dissociate from the complex when the complex moved away from the free ATPase into a medium containing Mg^{2+} (Figure 4a); however, when the complex was sedimented through the sucrose gradient without Mg^{2+} (Figure 4b), there was no ATPase activity in the membrane zone other than the small residual amount initially present in the depleted membranes.

Comparison of Ribosomes and Depleted Ribosomes as Acceptors of ATPase. S. fecalis membrane ghost preparations contain a substantial amount of attached ribosomes but no tRNA (Nielsen and Abrams, 1964). When the membrane ghosts are processed to solubilize the ATPase some RNA remains in the depleted membranes.

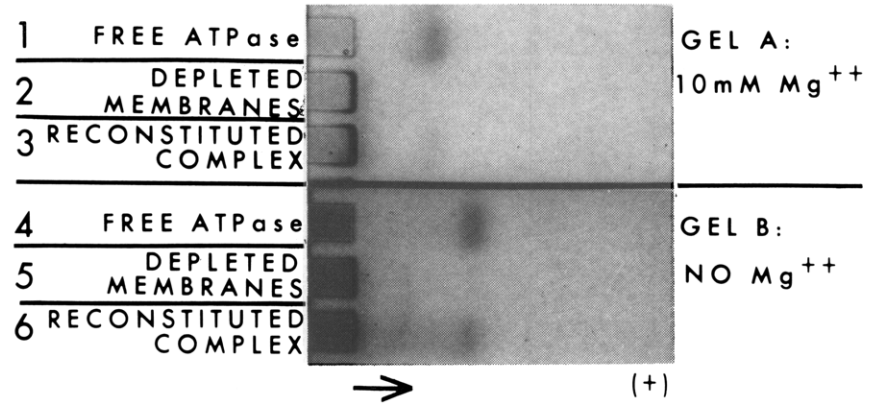

FIGURE 3: Electrophoresis of the ATPase–membrane complex in polyacrylamide gels with and without Mg^{2+}. A reconstituted ATPase–membrane complex was isolated by centrifugation of a mixture of 0.16 mg of depleted membranes, 0.91 unit of ATPase, and 10 mM Mg^{2+} (see Figure 1). The pellet was suspended in 0.1 ml of pH 8.5 buffer with 10 mM Mg^{2+}. Aliquots (30 μl) of the suspension were applied to gel A, slot 3 and to gel B, slot 6. As controls, we also applied the same amount of depleted membranes alone and free ATPase alone to gels A and B, as indicated. Gel A and its buffer compartment contained 0.0075 M Tris, 0.035 M glycine (pH 8.5), and 10 mM $MgCl_2$. Gel B contained 0.015 M Tris and 0.07 M glycine (pH 8.5). Gel A was run for 1.5 hr at 200 v and gel B was run for 1 hr at 300 v. Both gels were then stained for ATPase activity (Abrams and Baron, 1967).

Since the ATPase is highly anionic at pH 7.5 (Abrams, 1965), it was possible that some, if not all, of the binding of added ATPase to depleted membranes was due to a nonspecific Mg^{2+}-dependent bridge between the enzyme and the phosphate groups of RNA. To test this possibility a mixture of isolated ribosomes, free ATPase, and 0.02 M Mg^{2+} was analyzed by means of zone sedimentation in a sucrose gradient containing 0.02 M Mg^{2+}. The results are shown in Figure 4c. The same analysis with a comparable amount of depleted membranes instead of ribosomes is shown in Figure 4a. It is clear that very little enzyme, if any, became attached to ribosomes while a substantial amount became attached to the membranes. In other experiments preincubation of depleted membranes with ribonuclease did not affect the ability of the membranes to attach added ATPase. We may conclude, therefore, that the reconstitution of the ATPase–membrane complex is not due to binding to RNA. More importantly, the failure to bind to ribosomes emphasizes that the attachment of added enzyme to depleted membrane is at least to some degree selective.

Discussion

We have observed in these experiments the formation of an ATPase–membrane complex upon mixing the solubilized membrane ATPase and depleted membranes using three different techniques: ordinary centrifugation (Figures 1 and 2), zone electrophoresis (Figure 3), and zone sedimentation (Figure 4). While there can be little doubt that reattachment of the enzyme occurs it is important to assess its physiological significance. There are a number of characteristics of the recombination which support the contention that it represents an authentic reconstitution of a natural ATPase–membrane complex in which the membrane has a limited number of specific binding sites. (1) The depleted membranes become saturated with ATPase in the presence of a large excess of free enzyme; at saturation the amount of bound enzyme is only about 2% of the total membrane protein. Thus, only a very small part of the total membrane surface is involved in binding. (2) The artificial ATPase–membrane complex has a maximum specific enzyme activity which matches the specific activity of the native complex reasonably well. Moreover, the native membranes still containing their original complement of ATPase were unable to take up more enzyme when a large excess of free enzyme was added (Table I). This indicates that the sites occupied after recombination are the same as those in the native complex, as would be expected if the sites were specific. (3) The amount of enzyme bound at saturation was not increased by doubling the level of Mg^{2+}. This is also consonant with a limited number of specific binding sites. (4) Free ATPase did not bind significantly to ribosomes even in the presence of 0.02 M Mg^{2+}. This shows that the binding to membranes is selective (Figure 4c).

The reattachment of ATPase to the depleted membranes may be formulated as a two-step reversible process as follows

$$E + M \rightleftarrows E\text{–}M \qquad (1)$$

$$E\text{–}M + n Mg^{2+} \rightleftarrows \begin{matrix} [Mg^{2+}]_n \\ \diagup\diagdown \\ E\text{—}M \end{matrix} \qquad (2)$$

where E, M, and n symbolize, respectively, ATPase, a membrane binding site, and the number of Mg^{2+} atoms

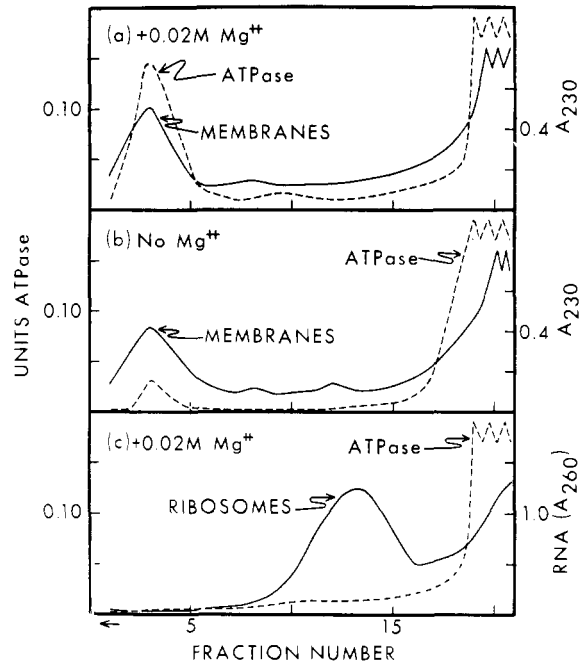

FIGURE 4: Effect of Mg^{2+} on zone sedimentation of a mixture of ATPase with membranes and with ribosomes; 0.2 ml of a mixture containing depleted membranes, 1.9 units of ATPase, 0.02 M Mg^{2+}, and pH 8.5 buffer (0.0075 M Tris and 0.035 M glycine) was layered on gradients a and b; 0.2 ml of a mixture containing 0.39 mg of ribosomes, 1.9 units of ATPase, 0.02 M Mg^{2+}, and pH 8.5 buffer was layered on gradient c. All gradients were 5–30% sucrose with a cushion of 0.8 ml of 0.9 saturated sucrose at the bottom. The gradients were buffered at pH 8.5. Gradients a and c contained 0.02 M Mg^{2+}; gradient b had no Mg^{2+}. Centrifugation was carried out at 5° at 118,000*g* in the SW39 head in the Spinco Model L2. Gradients a and b were run for 1.5 hr and c was run for 2 hr. Fractions (0.2 ml) were collected from the bottom of the tube and diluted with 0.8 ml of water. Absorbancy was measured as shown; 0.5-ml aliquots from every tube in the absorbancy peaks and from every other tube on either side of the peak were assayed for ATPase activity.

at a binding site. The first equation is based on the observation that recombination occurs in the absence of added divalent cations as shown in Figure 1, lower curve, and that it will dissociate when free ATPase is removed (Figures 3 and 4). The introduction of Mg^{2+} as an additional linkage between E and M, according to eq 2, is justified by the zone electrophoresis and zone sedimentation experiments showing that Mg^{2+} prevents dissociation of the complex in the absence of free ATPase (Figures 3 and 4). Furthermore, while Mg^{2+} stabilizes the ATPase–membrane complex it does not increase the maximum number of binding sites on the membrane (Figure 1). These considerations lead us to conclude that there are at least two types of interaction of an ATPase protein molecule at a single membrane binding site, one type involving Mg^{2+} and the other not. The interaction not involving Mg^{2+} may be hydrophobic but there is no evidence on this point. According to these findings one of the many functions of Mg^{2+} *in vivo* would be to preserve the attachment of ATPase to membrane. The importance of multivalent cations as stabilizing agents for complex cellular structures such as membranes and ribosomes is widely recognized. It is known from both *in vitro* and *in vivo* studies that in bacteria, Mg^{2+} ions specifically are required at a level of over 0.01 M to maintain the structure and function of ribosomes for protein synthesis (Marchesi and Kennell, 1967; Marshall *et al.*, 1967). As we have shown, such concentrations of Mg^{2+} obviously would likewise maintain the integrity of the ATPase–membrane complex. It is of interest to point out that beef heart and yeast mitochondrial membrane ATPase can be solubilized and reattached to submitochondrial membrane fragments (Kagawa and Racker, 1966; Schatz *et al.*, 1967). The effect of Mg^{2+} on attachment was not reported. The *S. fecalis* membrane has a very high content of cardiolipin, a polyanionic phospholipid (Ibbott and Abrams, 1964; Vorbeck and Marinetti, 1965). Since the ATPase is also highly acidic (Abrams and Baron, 1967), it is conceivable that Mg^{2+} forms an ionic linkage between the enzyme and cardiolipin.

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Sheep Brain 5′-Nucleotidase. Some Enzymic Properties and Allosteric Inhibition by Nucleoside Triphosphates*

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ABSTRACT: Enzymological studies of the action of inhibitors, adenosine triphosphate (ATP), uridine triphosphate (UTP), and cytidine triphosphate (CTP), upon partially purified preparations of sheep brain 5′-nucleotidase are described. The inhibition is of the mixed competitive and noncompetitive type with respect to adenosine monophosphate and is potentiated at high temperatures. Plots of kinetic data take the form of sigmoidal inhibition curves. Cooperative inhibitions are observed between inhibitor binding sites; however, first-order kinetics are observed in substrate saturation curves, suggesting the absence of interaction between substrate binding sites. Inorganic phosphate does not inhibit the activity of 5′-nucleotidase; at 0.2 mM, however, it reverses the inhibition by ATP, but not that by UTP and CTP. 5′-Nucleotidase is desensitized to ATP and UTP inhibition, but not to CTP inhibition, by treatment with *p*-mercuribenzoate.

The 5′-nucleotidases (5′-ribonucleoside phosphohydrolase, EC 3.1.3.5) comprise an apparently large and diverse group of enzymes, widely distributed in animal and plant tissues (Reis, 1934, 1951; Heppel and Hilmoe, 1951; Hurst and Butler, 1951; Kornberg and Pricer, 1960; Song and Bodansky, 1967).

An enzyme activity in the central nervous system, catalyzing the dephosphorylation of AMP[1] with a pH optimum around neutrality, was originally found by Reis (1951) in crude homogenates, and appears to be distinct from alkaline and acid phosphatase.

Our interest in brain 5′-nucleotidase was kindled by the observation of appreciable 5′-nucleotidase activity in crude sheep brain homogenates and by the inhibition exerted by ATP and other nucleoside triphosphates on the enzyme activity (Ipata, 1967).

The kinetic studies reported herein on the inhibition of 5′-nucleotidase by ATP, UTP, and CTP show that the inhibition is of an allosteric nature. The kinetics of 5′-nucleotidase *vs.* AMP is in accord with Michaelis–Menten theory.

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[1] Abbreviations used: PMB, *p*-mercuribenzoate; ATP, UTP, CTP, GTP, adenosine, uridine, cytidine, and guanosine triphosphates; AMP, adenosine monophosphate; IMP, inosine monophosphate.

Experimental Procedure

Materials. Nucleosides and nucleotides were obtained either from Sigma Chemical Co. or from Boehringer und Soehne. Whale skeletal myoglobin was obtained from Serevac Laboratories. Bovine serum albumin (containing serum albumin dimer), catalase, and pancreatic ribonuclease were obtained from Sigma Chemical Co. Adenosine deaminase was obtained from Boehringer und Soehne. Tris (Sigma) was used as a buffer in most experiments. Other chemicals were of reagent grade or of the highest quality available.

5′-Nucleotidase Assay Procedures. The enzyme activity was measured spectrophotometrically at 265 mμ by coupling the 5′-nucleotidase reaction to the deamination of adenosine formed, in the presence of an excess of adenosine deaminase. The standard reaction mixture contained, in a final volume of 1 ml, 0.033 M Tris-HCl buffer (pH 7.4), varying concentrations of substrate adjusted to pH 7.4, and about 100 μg of protein. Commercial adenosine deaminase (0.3 μg) was added to the reaction mixture, and the decrease in optical density at 265 mμ was followed with a recording spectrophotometer at room temperature. AMP was omitted in the reference cuvet. The velocity of the reaction was strictly proportional to the amount of 5′-nucleotidase up to rates higher than 0.200 absorbance unit/min. All rates studies reported here were conducted at rates of less than 0.080 absorbance unit/min.

Occasionally, the activity was measured from the