PRELIMINARY CHARACTERIZATION OF THE MEMBRANE-BOUND ADENOSINE TRIPHOSPHATASE OF *AZOTOBACTER VINELANDII*

A Thesis

Presented to

the Faculty of the Department of Biology

University of Houston

In partial fulfillment of

the Requirements for the Degree

Master of Science

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by

John Earl McEntire

August 1974

ACKNOWLEDGEMENTS

I wish to thank my advisor, Dr. Peter Jurtshuk, Jr., for his interest and guidance over my research. I also wish to acknowledge my wife, Diedra, and my daughter, Kim, for their understanding and support. This research was supported by the grant GM 17607-02, awarded to Dr. Peter Jurtshuk, Jr., from the National Institute of General Medical Sciences of the National Institutes of Health.

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ABSTRACT

The membrane-bound adenosine triphosphatase (ATPase) of Azotobacter vinelandii was examined and its direct relationship to the Azotobacter electron transport fraction (R_3) was established readily. Sonically disrupted Azotobacter cells were differentially centrifuged and distribution of ATPase activity was examined in particulate and soluble fractions. Highest specific activity for ATPase was consistently found in the R_3 fraction, a particulate fraction which sediments on ultracentrifugation at 144,000 x g for 2 hr. By increasing the interval time of sonication, the membrane-bound ATPase activity could not be solubilized nor released into the supernatant fraction. Optimal ATPase activity occured at pH 8.0; Mg^{++} ion when added to the assay was stimulatory, maximal activity occurring when the Mg^{++}:ATP stoichiometry was 1:1 on a molar ratio at the 5 mM concentration level. Sodium and potassium ions were shown to have no stimulatory effect. The reaction kinetics were linear for the time intervals studied (0-60 min). The membrane-bound ATPase in the R3 fraction was stimulated 12-fold by treatment with trypsin and fractionation studies showed trypsin treatment did not release ATPase activity from the membranous electron transport fraction. The ATPase was not cold labile and the temperature during the preparation of the R_3 fraction had no effect

on activity; overnight refrigeration at 4°C, however, resulted in a 25% loss of activity as compared to a 14% loss when the R_3 fraction was stored overnight at 25°C. A marked inactivation (60%) did occur by overnight freezing, and additional sonication failed to restore ATPase activity, indicating membrane reaggregation by freezing was not responsible for this type of inactivation. The addition of azide, ouabain, 2,4-dinitrophenol, or oligomycin to the assay system resulted in neither inhibition nor stimulation of the ATPase activity. The property of trypsin activation and the fact that ATPase activity is highest in the R_3 electron transport fraction suggests that its probable functional role is in coupling of electron transport to oxidative phosphorylation.

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LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
AVO	*Azotobacter vinelandii* strain O
DCCD	Dicyclohexylcarbodiimide
DEAE	Diethylaminoethyl
DNP	2,4-dinitrophenol
EDTA	Ethylenediaminetetraacetate
M.W.	Molecular weight
NAD^+	Nicotinamide-adenine dinucleotide
NADH	Reduced nicotinamide-adenine dinucleotide
PCMB	p-chloromercuri-benzoate
PCP	Pentachlorophenol
PEP	Phosphoenolpyruvate
Pi	Inorganic phosphate
Pr	Protein
Tris	Tris(hydroxymethyl)aminomethane-chloride buffer

INTRODUCTION

Detailed studies on bacterial adenosine triphosphatases (ATPase) have been performed with several genera of bacteria, i.e., Steptococcus (1-6,8,10,34,67,68), Micrococcus (7,16,50, 51,54,65,66,76,77), Bacillus (28,35,36,46-49,53,64), and Escherichia (15,18,19,29-31,39-43,55-56,58,62,63,69). A substantial amount of information is now available which suggests that ATPase activity in bacteria is bound to or closely associated with the cytoplasmic membrane. The specific function of the various bacterial ATPases is not completely understood, although it appears that this enzyme is associated with and can serve as a marker enzyme for several energy dependent processes like (a) ion transport (8), (b) coupling factor activity in oxidative phosphorylation and photophosphorylation (51,45), (c) association with ATP-dependent reactions such as NAD^+ transhydrogenase activity (39) and galactoside translocation (5), and (d) possibly other bioenergetic processes such as nitrogen fixation (11,32).

Depending on the organism, the bacterial ATPase may have many features in common with either the mitochondrial ATPase coupling factory or the eucaryotic $Na^+ + K^+$-activated ATPase.

Generally, the bacterial ATPases are Mg^{++} or Ca^{++}-stimulated enzymes which show little or no activation response to Na^{+} and/or K^{+} ion. They may be cold labile (when they are removed from the membrane) in soluble form (1,16), and some are affected by treatment with proteases (52). They are not generally sensitive to oligomycin, ouabain, or 2,4-dinitrophenol. Azotobacter vinelandii, a Gram-negative, aerobic, free-living nitrogen-fixing bacterium could theoretically contain an ATPase (or ATPases) associated with ion transport, coupling, transhydrogenation, nitrogen fixation, or with a variety of other energy linked reactions that might, for example, be associated with the contractile properties of the "microtubule" ultrastructure known to be present intracellulary as well as in isolated membrane fractions (59). Recent studies directed at examining the membrane-bound ATPase associated with oxidative phosphorylation in Azotobacter gave only fragmentary data concerning its major properties (17). Since little is known about the Azotobacter ATPase, and since bioenergetically this organism is of immense interest because of its unusually high respiratory capability which may be related to its efficient free-living nitrogen-fixing potential, and since ATPases are now commonly considered to be valid marker enzymes for numerous bioenergetic processes, a study was undertaken in order to characterize the nature of

this activity in membrane fractions and establish its functional relationship to the electron transport system of Azotobacter vinelandii strain O.

The following information is presented in order to familiarize the reader with the research which has been published on bacterial ATPases with emphasis on: (a) functional and biological significance of the bacterial ATPase, (b) techniques of isolation, purification, ultrastructural localization, and assay, and (c) description of physical and chemical properties of the enzymes.

Biological Function

Transport ATPase. As mentioned previously, functions related to ATPase activity are not limited to bacteria, but instead, appear to closely approximate similar complex enzyme systems of mitochondria as well as the plasma membranes of eucaryotes; the notable exception being the lack, thus far, of a bacterial counterpart to the contractile ATPases of muscle and plant tissues.

The most definitive functional studies thus far performed on a bacterial ATPase have been those of the transport

enzyme of Streptococcus faecalis. This is due, in part, to the fact that S. faecalis is an obligately fermentative microaerophilic organism which lacks an electron transport system, thereby eliminating the possibility of the enzyme's functioning in coupling of electron transport to oxidative phosphorylation. Definite proof of the ion transport function has been shown. Abrams and Smith (5) showed that when Streptococcus faecalis was grown in media having limiting K^+ concentration, the amount of the ATPase increased 1.5-2.1 fold, while two other membrane-bound enzymes tested did not increase. Cells grown under these conditions also exhibited increased rates of K^+ ion uptake. This induced ATPase and increased K^+ ion uptake relationship tended to substantiate a K^+ ion transport function for the enzyme. N,N'-dicyclohexylcarbodiimide (DCCD) inhibited not only ATPase activity but a number of energy-linked transport processes including the accumulation of K^+ ion by exchange for H^+ and Na^+ ions (34). Abrams and co-workers (6) have also reported genetic evidence which suggested that energized uptake of K^+ ion and cycloleucine is coupled to membrane ATPase activity. Mutants were isolated which were resistant to DCCD. These mutants also exhibited energized uptake of K^+ ion and cycloleucine in the presence of DCCD, an activity inhibited by DCCD in the wild type organism. Redwood (60) using solubilized ATPase from Streptococcus faecalis was able to demonstrate that the conductance of a bilayer

prepared from either natural or synthetic phosphatidyl choline was increaed by a factor of 10^2 to 10^4 depending on the presence of Mg^{++} and other cations, which indicated that the artificial bilayer-ATPase complex was similar to membrane-bound ATPase having ion transport function. This hypothesis was substantiated when the experiments were repeated with the highly purified S. faecalis enzyme (61). The S. faecalis enzyme appears to function by creating a proton-motive force, i.e., the ATPase "drives" ions along the membrane which results in a chemiosmotic-protein gradient (8).

In E. coli, there are many conflicting reports on the role of ATPase in transport of molecules. In general, it appears that ATPase is not obligatory for energy-dependent transport but can, under anaerobic conditions, accomplish energy-dependent transport via ATP hydrolysis (56).

Of all the bacterial ATPases studied, none have consistently shown the characteristics associated with the typical Na^+ K^+-stimulated transport ATPase of plasma membranes as defined by Skou (70). Exceptions to the above include the Staphylococcus aureus ATPase which is stimulated up to 65% by Na^+ or K^+ ion (26); early studies (29) also indicated the presence of a Na^++ K^+-stimulated ATPase in E. coli, however, the same authors sub-

sequently reported this activity to be only a small percentage of the total E. coli ATPase activity (30). There is also a report of Na^+ and K^+ stimulation of an ATPase in B. subtilis (64), this enzyme requiring high concentrations of Na^+ (75mM) and K^+ (90mM) ions in order to stimulate activity 70% and 90% respectively. It should be emphasized that these examples of Na^+ and/or K^+ stimulation of bacterial ATPase activities are exceptions. In most studies, even those employing the same organism, Na^+ and K^+ ions were shown to have no effect and in many instances these cations were inhibitory for ATPase activity. There has been no consistent report, to date, of a classical plasma membrane-type transport ATPase (Na^++ K^+-stimulated, Mg^{++}-requiring, ouabain-sensitive) in bacteria.

Coupling factor ATPase. Unlike S. faecalis, most organisms studied contain potentially multifunctional ATPase(s), i.e., associated with coupled electron transport activity, or related to transport mechanisms, or possibly associated with a variety of other energy-dependent reactions which might well require enzymatic hydrolysis of ATP. ATPases which function in coupling of electron transport to phosphorylation are indicated in those organisms which carry out oxidative phosphorylation, photophosphorylation, or "anaerobic respiration" (electron transport in which the terminal acceptor is a molecule other than oxygen).

The most extensively studied bacterial enzyme implicated as a coupling factor in oxidative phosphorylation has been the ATPase of Micrococcus lysodeitkticus. Munoz, Salton and co-workers have shown that the Micrococcus enzyme is similar in many respects to the mitochondrial ATPase known to function in coupling (51). This enzyme generally has latent activity when associated with the membrane and is activated when treated with trypsin (50), once solubilized it may not exhibit such activation. Trypsin activation, however, may depend on the method of preparation of the particles (or membrane fraction) as will be discussed in a following section. The Azotobacter vindelandii ATPase has the ability to serve as a coupling factor in oxidative phosphorylation. It, also, is trypsin-activated although it does not appear allotopic in its response to trypsin when in the soluble form (17). Based on the similarity of its properties to other bacterial coupling factor enzymes, the probable function of the B. megaterium ATPase may also be coupling, although preparations have not yet been found which can catalyze coupled phosphorylation (35,36).

The latency of ATPase activity, a commonly encountered property of those enzymes thought to be involved in coupling may indicate an in vivo function that is the direct opposite of hydrolysis of ATP, i.e., phosphorylation of ADP. Since ATPase activity is increased markedly by treatment with trypsin (or

heat), a conformational regulatory mechanism may be involved. The trypsin may hydrolyze an inhibitory protein component which intracellularly does not allow ATP hydrolysis to occur. Once trypsinized, the control protein can no longer function in ATP synthesis and the ATPase now acts as the catalyst, allowing the thermodynamically favored reaction, ATP hydrolysis, to occur. This is the most likely explanation for the trypsin activation of ATPase activity and may involve further changes in membrane conformation (33).

Genetic evidence of the role of ATPase in coupling has been demonstrated in E. coli. Cells which were respiratory-deficient and neomycin-sensitive (due to their inability to generate enough energy to concentrate neomycin intracellularly to toxic levels) were studied. These organisms could grow with glucose as a carbon source (indicating fermentation), but were unable to grow on Kreb's cycle intermediates even though they were able to oxidize these compounds. This apparent lack of coupling ability was accompanied by absence of the E. coli ATPase (40).

Coupling factor ATPase activity for photophosphorylation reactions has been studied in the photosynthetic bacteria Rhodopseudomonas and Rhodospirillum. Photophosphorylation

activity, in extracts of Rhodopseudomonas capsulata, was decreased after sonication but could be restored by addition of soluble factors released by sonic oscillation which contained ATPase activity (9). This ATPase has since been shown to be light-activated (45). After brief exposure to light, specific activity increased approximately 2-fold although the K_m for ATP remained constant. Classical uncouplers, which stimulate the ATPase activity in the dark, have no effect on the light-activated enzyme. Sonication and subsequent removal of supernatant fractions of Rhodospirillum rubrum chromatophores reduces photophosphorylation and Mg^{++}-ATPase activities of the particulate fraction by 50-85%. Both of these activities are recovered proportionately to the amount of supernatant replaced. When the volume of supernatant added exceeded the original amount, the activities become greater than that of the untreated chromatophores (37). This coupling factor ATPase has been shown to function specifically in the terminal step of ATP synthesis (25).

Marunouchi (44) has separated a Mg^{++}-requiring $SO_3^{=}$-stimulated ATPase from the Mg^{++}-ATPase in the organism Thiobacillus thioxidans. This organism has an electron transport chain that can utilize sulfite as the terminal electron acceptor. It is capable of phosphorylation and it is suggested that the $SO_3^{=}$-stimulated ATPase in this organism

serves as a coupling factor. Similarly, Guarraia and Peck (27) have reported a dinitrophenol-stimulated ATPase in extracts of Desulfovibrio gigas, an organism capable of "anaerobic respiration" by its ability to convert sulfite to sulfide or fumarate to succinate. They also suggest that this ATPase acts as a coupling factor.

Transhydrogenase ATPase. Another biological function of ATPase is its involvement in the ATP-driven NAD^+transhydrogenase. Kanner (34) has isolated E. coli mutants deficient in ATPase which are unable to utilize ATP to drive the NAD^+ transhydrogenase reaction. Konings, using Rhodospirillum rubrum chromatophores that were depleted of ATPase and transhydrogenase activities, was able to restore ATP-driven transhydrogenase only after reconstitution with both the ATPase factor and the transhydrogenase factor (42).

RNase and DNase - ATPases. The relationship of ATPase to RNase and DNase has recently been reported. Since ATPase is almost always a membranous component, the association of membrane to nuclear material as well as membranous structures associated with division septae may eventually be related to ATPase association with nucleases. Paetkau (55) in purifing the DNA -dependent RNA polymerase of E. coli found a 68,000 M.W. ATPase associated with the active enzyme which was difficult to remove from the polymerase.

Eskin (18) reports that the E. coli restriction endonuclease hydrolyzes ATP in the presence of 5-adenosylmethionine and DNA with unmodified restriction sites, while Zinder (78) shows the ATPase activity of restriction endonuclease B is effected by DNA length. In purifying the B. subtilis ATP-dependent DNase, Ohi (53) has shown that a DNA-dependent ATPase is inseparable from the purified DNase.

Nitrogenase ATPase. ATPase activities have been reported associated with the nitrogenase systems of some organisms, but whether these really represent true ATPases is questionable. Hardy (32) reported a reductant-dependent ATPase in Azotobacter, but Bulen states that no classical ATPase activity can be found in the nitrogenase system, and that reductant-dependent ATP hydrolysis proceeds only in the presence of nitrogenase enzymes I and II and $S_2O_4^{=}$. Therefore, ATP hydrolysis does not appear to be due to a single enzyme (11), but does occur with the reconstituted nitrogenase system. ATP is required as a functional component in all purified enzyme systems carrying out the basic nitrogen fixation reaction ($N_2 \longrightarrow NH_3$).

Other possible ATPase functions. Numerous other energy-requiring reactions which have ATPase activity associated with them in eucaryotic organisms have not yet been studied in detail in baceteria. When one considers the known fuctions of ATPase

in other systems, the applications to bacterial systems become evident. Do ATPases associated with contractile processes in muscle and plant tissue have a bacterial counterpart? This possibility is suggested by the presence of microtubles in Azotobacter as reported by Pope and Jurtshuk (59) which may have contractile properties. Recent localization studies show that ATPase concentrates at the base of bacterial flagella (73), suggesting possible contractile or locomotor function. Is bacterial ATPase activity associated with the cell cycle as in sea urchin eggs (47) where ATPase shows two peaks of activity and is effected by temperature as in the cell cycle? Until the ATPases of other organisms are isolated and more highly purified, it is difficult to discuss the heterogenicity of ATPase activity. Even then, as suggested by Evans (19), ATPase may be a single enzyme which may function differently depending on physical variables and the type of bioenergetic process involved.

ATPase Assay

Assay for ATPase activity is relatively simple; one needs only to measure the release of inorganic phosphate (Pi) since this and adenosine diphosphate (ADP) are the sole products of the ATP hydrolysis, the reaction catalyzed by ATPase.

There are a number of methods for this assay, the most commonly used being the analysis of Pi by the method of Fiske and Subbarow (20). This method measures colorimetrically the inorganic phosphate released from ATP hydrolysis. The method of Fritz and Hamrick (21) is a spectrophotometric procedure which involves measuring NADH oxidation in the following series of coupled reactions:

$$\text{ATP} + H_2O \xrightarrow{\text{ATPase}} \text{ADP} + \text{Pi}$$

$$\text{ADP} + \text{PEP} \xrightleftharpoons{\text{Pyruvate Kinase}} \text{ATP} + \text{Pyruvate}$$

$$\text{Pyruvate} + \text{NADH} \xrightleftharpoons{\text{Lactic Dehydrogenase}} \text{Lactate} + \text{NAD}$$

The rate of decrease in optical density at 340 nm is a direct measure of ATPase activity when the pyruvate kinase and lactic dehydrogenase are present in excess. This method is advantageous since the ADP (often an inhibitor of ATPase activity) is removed continually as ATP is regenerated, and therefore, substrate concentration does not become limiting. Assays in which the terminal phosphate of ATP is radioactively labelled are used by some investigators (31).

Isolation and Purification

The simplest and most widely used procedure for ATPase isolation starts with obtaining bacterial protoplasts (or

spheroplasts) or "membrane ghost" fractions. In the case of Gram (+) cells, protoplasts may be formed by suspending the cells in lysozyme and appropriate buffers which completely removes cell wall material leaving an osmotically sensitive protoplast. This protoplast is then treated by addition of 0.01 M glucose which causes rapid swelling and lysis of the protoplast due to a change in permeability and influx of an osmotic stabilizer (4). The membrane ghosts thus formed may then be sedimented and washed, and assays for enzyme activity may be carried out. It is important, however, that Mg^{++} ion concentration be carefully monitored. In the case of the Gram (-) cell, due to the composition of its cell wall, it is necessary to add lysozyme and ethylenediaminetetraacetate (EDTA) for the formation of the spheroplasts, which is then followed by similar osmotic lysis and sedimentation (19). Although ATPase activity may be conveniently studied using such membrane preparations, for further purification it is necessary to solubilize the enzyme. Abrams and co-workers have shown that the enzyme of Streptococcus faecalis is solubilized by repeated washing of the membrane ghosts with buffer deficient in Mg^{++}ion (1). It is of interest that ATPase is not released during the initial six washings with Mg^{++}-deficient buffer, but during the seventh and eighth washings the enzyme is released abruptly and obtained quantitatively in relatively pure form and high yields.

Therefore, the release of ATPase in these washings is highly specific rather than a general solubilization of membrane protein. It also suggests that Mg^{++} ion is involved in the binding of the enzyme to the membrane and that as the Mg^{++} ion is diluted out to a certain critical concentration level, the enzyme is released. The structural significance of Mg^{++} ion will be discussed in detail with information presented on the enzyme structure. Voelz (75) has demonstrated this phenomena with electron microscopic cytochemical localization studies. Cytochemical localizations of various phosphatases are based on the Gomori reaction (23) whereby partially fixed tissue is incubated with a specific substrate in the presence of lead or calcium ion. Upon enzymatic cleavage of a phosphate group, an insoluble precipitate of either lead phosphate or calcium phosphate is formed at the site of reaction. Voelz has shown that Myxococcus xanthus ATPase is located on (or within) the cytoplasmic membrane, and as repeated localization studies are carried out on cells washed in Mg^{++}-deficient buffers, the precipitate (and therefore ATPase activity) is shown to "migrate off" the membrane, through the cell wall, and finally is not seen in the cell but can be demonstrated in the washing fluid. The locale of the acid and alkaline phosphatases appear to be unaffected by similar washes, showing the specificity involved in the release of ATPase activity.

Munoz and co-workers partially purified the ATPase of Micrococcus lysodeikticus by a procedure based on the enzyme's association-dissociation properties with the membrane. The enzyme was released from membrane ghosts by a similar repeated washing procedure (50).

ATPases can be further purified in a number of ways. The S. faecalis enzyme, once released could be centrifuged on a sucrose density gradient and yielded a reasonably pure preparation. However, yield was so small (1 mg or less) that the extensive studies needed for physical and chemical characterization could not be undertaken. Schnebli and Abrams devised a purification scheme whereby they could prepare 10 to 20 mg of pure ATPase in about a week's time. This method (67) consisted of heat treatment, gel filtration, and repeated chromatography on diethylaminoethyl (DEAE) cellulose as follows: the cell ghosts were washed as mentioned earlier with Mg^{++}-deficient buffers to release ATPase from the membranes. The washes, containing the solubilized ATPase, were pooled and 10 mM Mg^{++} ion was added. This usually resulted in the precipitation of the enzyme. RNase was added to remove contaminating RNA, followed by dialysis overnight against TM buffer, pH 7.5. The material was then heated to 55° for 10 min and a white inactive precipitate was removed by centrifugation and discarded. The heat-treated extract was placed on a DEAE-cellulose column where

all the ATPase was adsorbed. The ATPase was then eluded using a KCl linear gradient at about 0.27 M KCl. The enzyme was then precipitated with 85% saturated ammonium sulfate, centrifuged, dialyzed and then fractionated on Agarose A. Chromatography on DEAE-cellulose was repeated, followed by precipitation and dialysis as before. By this procedure the enzyme was purified 50-fold over that released from the membrane ghosts at a yield of 25-30%. This purification enabled these investigators to characterize the S. faecalis ATPase extensively as will be discussed later.

Munoz, et al, (51) were able to further purify the soluble M. lysodeikticus ATPase after concentration by reverse dialysis against 0.03 M Tris buffer. This concentrate was then placed on a Sephadex G-200 column, fractions were collected and assayed, and reapplied to another Sephadex column for a total of four times. This method also resulted in an approximate 50-fold purification. Andreau and Munoz (7) used a similar procedure on the same organism except they substituted EDTA-ammonium bicarbonate for Tris buffer in the original washing procedures and recovered an enzyme that was not stimulated by trypsin. More recently, Salton and Schor have been able to purify this enzyme by extraction with n-butanol and the purified ATPase also shows the loss of trypsin activation (65).

Using similar methods, the E. coli (31) and B. megaterium (46,28) ATPases have also been purified. Mirsky claims to have developed a faster method for purification using a glycerol gradient (47).

Physical and Chemical Properties

All the bacterial ATPases studied thus far require a divalent cation, to some degree, for activity. The cation is normally Mg^{++} or Ca^{++} ion although other divalent cations can often be substituted with only a slight loss in activity. The S. faecalis ATPase (1) requires Mg^{++} ion in a 1:1 ratio to ATP for maximum activity at pH 7.5. Na^{+} and/or K^{+} ions have little effect. Activity is inhibited by ADP and Pi, DCCD, Dio 9, and guanidines, while oligomycin and ouabain have no effect. Harold and Baarda (34) have found the ATPase of S. faecalis to be inhibited by DCCD when membrane bound but unaffected in the soluble form. The inhibited membrane-bound ATPase was reactivated when the enzyme was released from the membrane. This indicated that DCCD reacted with some component of the system other than the enzyme, possibly by indirectly changing its conformation. DCCD also inhibited a number of other energy-dependent transport processes. It is believed that DCCD inhibition of transport is the direct result of the inhibition of the ATPase activity. However, it was

also suggested that DCCD might bind to the membrane so as to indirectly inhibit both the hydrolysis of ATP and energized transport. It was later found that a specific protein, carbodiimide-sensitizing factor (CSF), was responsible for this effect (3). The DCCD is thought to react with this protein, thereby transferring its inhibitory effect, via conformational changes to the bound enzyme while not affecting the solubilized enzyme. In mutant DCCD-resistant cells (6), reconstitution hybrids showed that the resistance was due to a modification of the CSF protein and that enzyme, nectin (to be discussed later), and other membrane components were unaffected. This difference in properties of the bound and solubilized enzymes is termed allotopy and is reported in other bacteria as well as in mitochondria. Both the bound and solubilized enzymes are competitively inhibited by ADP and Pi with Ki values of 0.7 and 10 mM, respectively, and synergistic effects are observed in the presence of both. The eucaryotic transport ATPase is known to involve phosphorylated intermediates, and therefore an attempt was made to demonstrate such in the Streptococcus system. Labelling experiments failed to show any phosphorylated intermediates and also eliminated ADP-ATP and Pi-ATP exchange mechanisms. The findings that both end products (Pi and ADP) were competitive inhibitors and that together their effect was more than additive, as well as failure to observe phosphorylated

intermediates or exchange reactions suggests "Rapid Equilibrium Random Bi Bi" reaction kinetics according to the terminology of Cleland (14). According to this mechanism, all possible enzyme-substrate and enzyme-product complexes dissociate quickly and reversibly. The rate-limiting step is the conversion of the enzyme-substrate to the enzyme-product complex. There is also no definite order in which the products leave the enzyme site. This accounts for both products being competitive inhibitors of the enzyme when tested separately. The mechanism of synergistic inhibition is unexplained. It is possibly due to the fact that in the presence of Pi, the ADP can bind to both the free enzyme and the enzyme-Pi complex. The ATPase from Micrococcus lysodeikticus has also been shown to have no ADP-ATP exchange (51). Schnebli (67) has shown that the Streptococcus enzyme, which is composed of 12 subunits and exhibits classical kinetics, has no homotropic cooperativity between multiple active sites, or that there is only one active site per molecule since the Hill plot of varying concentrations of substrate gives an interaction coefficient such that the slope of the line n = 0.94. No detailed mechanism except that indicated above has been proposed for the hydrolysis of ATP by ATPase in a bacterial system to date.

Extensive structural studies also have been performed by Schnebli and co-workers on the Mg^{++}-ATPase of Streptococcus

faecalis (68). Sedimentation equilibrium experiments were done in a Yphantis cell and adjustments were made in order to use the meniscus depletion method. For calculation of the average molecular weight, slopes of linear plots of the natural logarithm of the corrected fringe displacement (f) against the radial distance squared (r^2) were used with the following formula:

$$M.W. = \frac{2RT}{(1-vp)w^2} \cdot \frac{d \ln f}{d r^2}$$

where R is the gas constant, T is absolute temperature, v is partial specific volume, p is density of the solvent and w is angular velocity. Sedimentation velocity was determined to be 13.4 at 20° in water. Partial specific volume was found to be 0.742 ml per gram compared to 0.735 ml per gram calculated from the amino acid composition. The average molecular weight was found to be 385,000 and was independent of the initial protein concentration, which is an indication of the homogeneity of the enzyme preparation. A maximal turnover number of 18,000 per molecule per min at 38° was calculated. A frictional ratio of 1.35 was calculated from the sedimentation constant and the molecular weight. The molecular weights of the ATPase subunits were 33,000 as determined by sedimentation studies in guanidine hydrochloride; the value calculated by amino acid composition was 32,800. Since the molecular weight of the enzyme is 385,000 it was concluded that the ATPase was made up of 12 polypeptide

chains with molecular weights of 33,000. Cysteine was determined after performic acid oxidation and was found to occur in the lowest frequency and the molecular weight calculated per half-cystine was determined to be 32,800 as mentioned above. No. NH_2-terminal groups could be detected by ^3H-fluorodinitrobenzene and the investigator concluded that the NH_2-terminal acids were proline, tryptophan, or cystine which would not have been detected by that method, or that the NH_2-terminal amino acid was blocked by some other constituent. The COOH-terminals were not examined. It was also reported that the subunits were not identical. Two bands, designated α and β, which differ in their response to reducing agents during electrophoretic analysis were detected. Electron micrographs of the purified enzyme show particles of uniform size which are made up of six globules surrounding a hole, and the investigators proposed a structure of twelve polypeptide chains, six α and six β, with one α and one β forming together each of the six globules which are arranged in hexagonal form. These investigators also claimed that the corresponding cavities in micrographs of the depleted membranes were the original sites for the binding of the ATPase molecules. The proposed ATPase model would have the correct frictional ratio of 1.35 as determined for the pure enzyme. As mentioned previously, Mg^{++} is related to the attachment of the enzyme to the membrane. Abrams and Baron (2) have shown that the solubilized ATPase will recombine quantitatively with

depleted membranes in the presence of Mg^{++} ion. The amount of recombination was dependent on the amount of free ATPase in the system and approached a maximum when ATPase was in great excess. The addition of Mg^{++} ion did not increase the number of ATPase molecules recombining with the membrane, but strengthened the bonds. In the absence of Mg^{++} ion, the reconstituted ATPase-membrane complex dissociated when no free ATPase was present in solution. In the presence of Mg^{++} ion, the complex did not dissociate. The authors concluded at this time that there were a limited number of specific binding sites on the membrane for reversible attachment of ATPase, that two types of interaction between the membrane and ATPase were present and one of these involved Mg^{++} ion, and that Mg^{++} ion provided additional linkages between the enzyme and the membrane. They also proposed, from studies on the reconstituted ATPase system, that in the native state the enzyme made up about 2% of the total protein. The sites were specific for ATPase and the reconstituted enzyme attached at exactly the same sites as were occupied by the native enzyme. They also proposed the following mechanism for reattachment of the enzyme to the membrane, where E, M, and n are respectively, ATPase, membrane binding site, and number (of Mg^{++} ions):

$$E + M \rightleftharpoons (E - M)$$

$$(E - M) + nMg^{++} \rightleftharpoons \begin{matrix} (Mg^{++})_n \\ \diagup \quad \diagdown \\ (E - M) \end{matrix}$$

The first equation explained how reconstitution occurred in the absence of Mg^{++} ion while the second equation showed how Mg^{++} ion could strengthen the bond. They also showed, the ATPase to be highly acidic and that the membrane had a high concentration of cardiolipin, a polyanionic phospholipid, and suggested that this formed the ionic linkage which bound the enzyme to the membrane via the Mg^{++} ion. It is important to note that these reconstitution experiments were carried out prior to the complete purification of the ATPase which was outlined above. After the enzyme was purified, it was found that reconstitution did not occur even in the presence of Mg^{++} ion. A protein termed "nectin" (from Latin nectare, meaning "to bind") of molecular weight 37,000 was isolated and found to be necessary for the reconstitution of the enzyme-membrane complex (10). Mg^{++} ion, as before, was thought to strengthen the bond. The amount of ATPase which attached to the depleted membrane in the presence of Mg^{++} ion was found to be directly related to the amount of nectin present. A temperature-sensitive reaction between nectin and ATPase takes place prior to attachment to the membrane. The activity of the reconstituted nectin-Mg^{++}-ATPase-membrane complex is identical to that of the native enzyme.

Many of the properties of the E. coli ATPase differ in various reports. Davies and Bragg (15) using a solubilized enzyme prepared similarly to that used by Abrams, report a Ca^{++} or Mg^{++} ion requirement at pH 9.5 with optimal cation:ATP

averaging about 0.5. In the presence of Ca^{++} ion the enzyme is specific for ATP, while in the presence of Mg^{++} ion there is some hydrolysis of ADP. The E. coli enzyme is cold labile but addition of 20% (v/v) glycerol stabilized the activity. The molecular weight is reportedly between 365,000 and 390,000. Na^{+} and K^{+} ions are inhibitory. ADP inhibits the Ca^{++}-ATPase activity but is hydrolyzed by the Mg^{++}-ATPase. Optimal pH is 8.5-9.5 and energy of activation is 20.7 kcal/mole. Kobayashi, using a similar preparation reports a molecular weight over 400,000, similar stability and cation ratios. The author does not comment on apparent substrate inhibition observed in some of his figures. ADP was a competitive inhibitor with Ki = 3×10^{-4} M. Azide, DCCD, and guanidine were inhibitory while oligomycin, KCN, DNP and ouabain had no effect (41). Using membrane fragments of E. coli, Evans (19) showed that the ATPase could be activated by Ca^{++} or Mg^{++} ion at different pH. At pH 9.0 optimal activity occurred in the presence of Mg^{++} ion at a Mg^{++}:ATP ratio of 0.4, while at pH 9.9 optimal activity occurred in the presence of Ca^{++} ion at a ratio of 0.2. Both Ca^{++} and Mg^{++} ATPase were inhibited by azide, DCCD, and PCP. Ca^{++}-ATPase activity was inhibited by Na^{+} and K^{+} ions, as well as Li^{+}, Rb^{+}, and Cs^{+} ions. Evans suggests that the E. coli ATPase is a single protein which is activated by either Ca^{++} or Mg^{++} ion at different pH. It would appear that this ATPase might be involved in some type of regulatory

mechanism whereby its properties differ depending on cellular pH. Hanson (31) has purified the E. coli ATPase by treatment of sonically prepared particles with Triton X-100, and reports a molecular weight of 360,000 with subunits of 60,000; 56,000; 35,000; and 13,000. The enzyme breaks down into these subunits as it becomes inactivated by cold. It is activated by Mg^{++} or Ca^{++} ion; Co^{++}, Ni^{++}, and Mn^{++} ions activate slightly. Azide and Pi are non-competitive inhibitors. An antibody prepared to the purified ATPase inhibits the enzyme in its soluble and bound states and also inhibits all associated functions such as the ATP-driven NAD^{+} transhydrogenase reaction. An ATP-ADP exchange reaction reportedly takes place in E. coli (62). This exchange is stimulated by high concentrations of Mg^{++} ion or Pi; it is also stimulated by azide, and unaffected by DCCD and Dio 9.

The purified ATPase from B. megaterium strain KM (46,48) was shown to have a molecular weight ranging from 379,000 to 410,000 with subunits of 68,000 and 65,000. It is activated by Ca^{++} ion preferentially to Mg^{++} ion, and Ca^{++}:ATP ratio can vary from 1:5 to 1:1 with no change in activity. Na^{+} and K^{+} ions have no effect. ADP is a competitive inhibitor and the enzyme is slowly inactivated at 4° in both soluble and bound forms. Studies with antibody

prepared against the ATPase suggest that the enzyme is located on the inner membrane with some exposure to the outer membrane (49). Ishida (35,36), using another strain of B. megaterium solubilized the ATPase by dialysis and mild alkalai treatment. The bound enzyme was activated with either Ca^{++} or Mg^{++} ion while the soluble enzyme was activated only by Ca^{++} ion. Dithionite stimulated the bound enzyme 2-fold but stimulated the soluble enzyme 7.5-fold. Gramicidin S stimulated the soluble enzyme but inhibited the bound form. The purified ATPase of the thermophillic organism B. stearothermophillus (28) has a molecular weight of 280,000 and is both thermostable and cold stable, with optimal activity occurring at 65 °.

The ATPase of M. lysodeikticus has a molecular weight varying from 330,000 to 350,000. It is trypsin activated and was known to contain subunits (51). A trypsin insensitive enzyme was prepared and had subunits designated α, β, γ, and δ with molecular weights of 52,000; 45,000; 41,500; and 28,500, respectively, and a suggested formula for its composition was $\alpha_3 \beta_3 \delta(\gamma)$. The enzyme requires Ca^{++} or Mg^{++} ion and is not affected by Na^{+} or K^{+} ions. It appears to be cold stable. It is inhibited by azide but not by oligomycin or ouabain. Electron micrographs of negatively stained preparations show a central unit surrounded by six identical subunits and resemble the stalked particles of inner membranes.

Oppenheim and Salton (54) have shown by ferratin labelling that the M. lysodeikticus ATPase is on the inner face of the membranes and that neither ATPase nor headpieces are observed in the mesosomal membranes. Similar results were obtained by iodination with 125_I (66). These studies are in contrast to other investigations which show a high concentration of ATPase in the mesosomes of some bacteria (22,72). Whiteside, who succeeded in preparing antibody to the ATPase of M. lysodeikticus (76) has attempted to show evolutionary relationships between groups of bacteria by reacting their ATPase-containing membranes with this antibody (77). This technique may have significance in relating functional types of enzymes as well as demonstrating differences between the enzymes of the same species.

Thacker (71) has shown the kinetics of ATPase activity in Pseudomonas aeruginosa to be sigmoidal and questions lack of sigmoidal kinetics in the reports of Abrams and Munoz where subunit structure is known to be present (68,51).

MATERIALS AND METHODS

Bacterial Strain and Growth Conditions

Azotobacter vinelandii strain O (ATCC #12518) was grown on Burk's nitrogen-free (NF) media with 1% acetate as sole carbon source. One liter batch cultures were grown at 30° for 24-30 hours and aerated by reciprical shaking (using low form culture flasks). These were used as seed innocula for large scale batch preparations. Large scale cultures were grown in glass carboys containing 14 liters of Burk's NF/acetate media. After 30 hours of growth at 30° with forced filtered aeration, the Azotobacter cells were harvested by a Sharples centrifuge; washed and standardized resting cell preparations were made in 25 mM Tris-HCl buffer, pH 8.0, as described elsewhere (38).

Preparation of Membrane Fractions

Turbidimetrically standardized resting cell suspensions were disrupted by a sonic oscillation procedure using a Heat Systems Ultrasonics Model M140 sonicator, power setting 7 (output 70) and sonicating for 2 minute intervals with adequate cooling for a total time of 10 min. As described in the text, varying sonication times were used in one study. After sonication, the disrupted homogenate was differentially centrifuged

at the speeds and times described in the flow diagram shown in Figure 1 using both a Beckman J-21B preparative centrifuge and a Beckman L3-50 ultracentrifuge. All procedures were carried out routinely at 4° and where indicated, studies on cold lability were carried out at room temperature (25°).

ATPase Assay

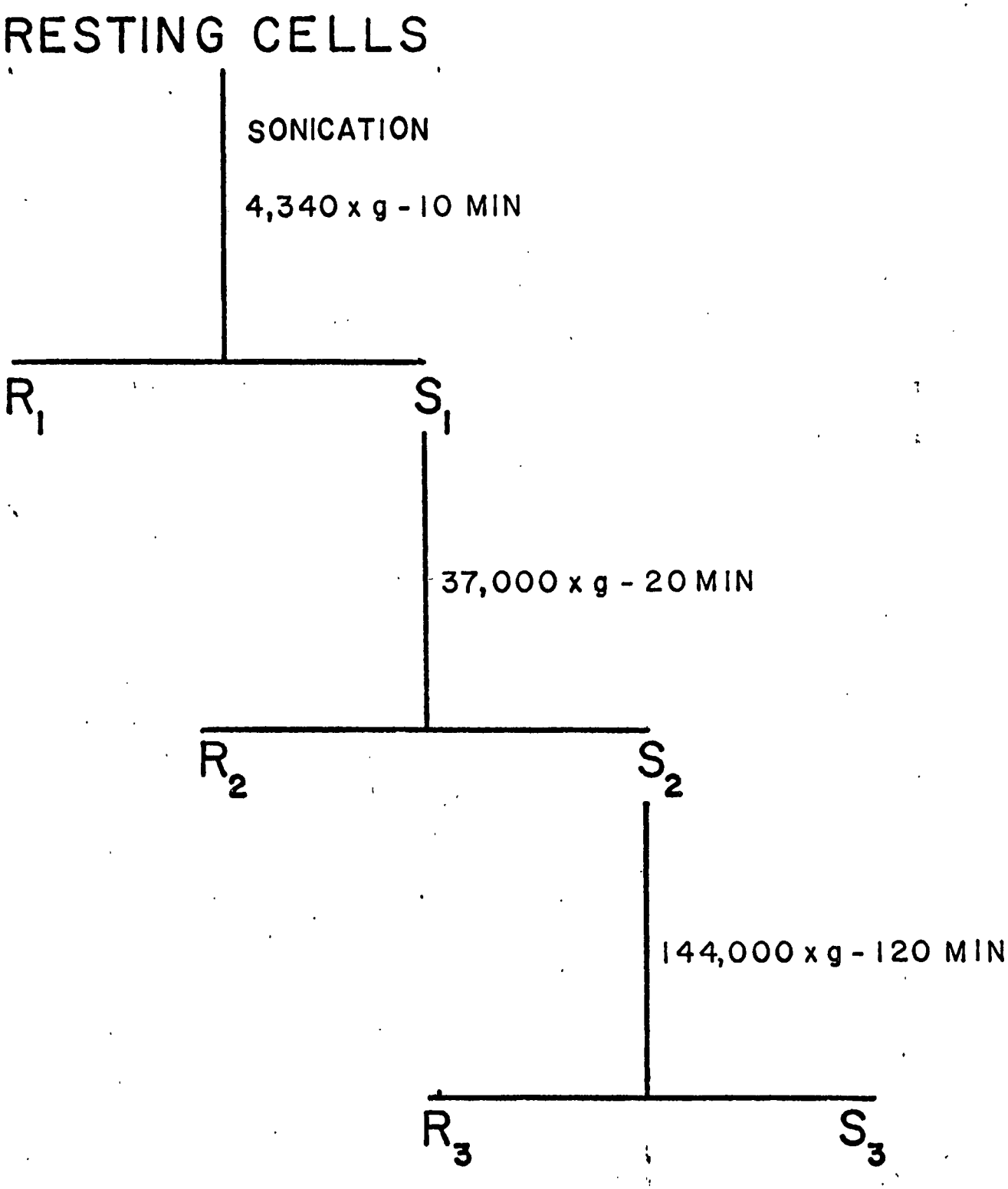
The standard colorimetric assay system was used for all ATPase determinations. The enzyme reaction mixture contained 25 mM Tris-HCl, pH 8.0; 5 mM NaATP; 5 mM $MgCl_2$; and 1-3 mg of protein in a total volume of 1.0 ml. After 10 minutes incubation at 30°, the reaction was stopped by the addition of 0.5 ml of 15% (w/v) trichloroacetic acid (TCA). Inorganic phosphate released from ATP hydrolysis was determined colorimetrically by the method of Fiske and Subbarow (20). Control tubes were prepared identically for each experimental system examined, however, in these tubes TCA was added prior to addition of the substrate ATP. All specific activities are expressed as umoles Pi (inorganic phosphate) liberated/min/mg protein.

Effect of Cations

Mg^{++} ion concentration was varied from 0-20 mM in the presence of varying concentrations of ATP to determine optimal

Figure 1. Fractionation scheme outlining the preparation of the Azotobacter electron transport fraction (R_3). The other residual fractions (designated "R") contain debris and unbroken cells (R_1) and large membrane fragments (R_2). The supernatant fraction (S_3) contains the soluble enzymes and very light membrane fragments that were not pelleted by the ultracentrifugation indicated.

PREPARATION OF A. VINELANDII ELECTRON TRANSPORT FRACTION (R_3)



Mg^{++}:ATP ratio. Mg^{++} ion was replaced in the standard assay system where indicated by other divalent and monovalent cations including Ca^{++}, Mn^{++}, Co^{++}, Hg^{++}, Cu^{++}, Zn^{++}, Na^{+}, K^{+}, and $Na^{+}+K^{+}$ ions to determine the specificity of the reaction for Mg^{++} ion. Final concentration of all metal ions was 5 mM.

Inhibitor Studies

Various inhibitors of ATPase activity and electron transport activity were added to the standard assay system in a final concentration of 10^{-4} M, except oligomycin, of which 5.0 micrograms were added to the system. Those inhibitors which markedly inactivated the ATPase were titrated at lower concentrations.

Trypsin Treatment

The Azotobacter R_3 electron transport fractions (protein concentration 10-30 mg/ml) were treated with trypsin (0.5 mg/mg R_3 protein) for 4 min at 25°. At the end of this time interval, an excess of soybean trypsin inhibitor (type II) was added. Controls were prepared by adding the trypsin inhibitor prior to addition of trypsin.

Protein Determination

Protein was determined by the biuret method using bovine serum albumin as a standard (24).

EXPERIMENTAL RESULTS

Association of ATPase Activity with Membranes of the Electron Transport Fraction (R_3)

It has previously been established that the Azotobacter R_3 fraction contains the highest activity as well as the greatest concentration of the membrane-bound electron transport enzymes. Initial ATPase activity studies showed that this activity was also membrane bound, and ATPase activity units also concentrated in this fraction, as shown in Table 1. A study was carried out to determine if increased sonication times would release the bound ATPase in the R_3 fraction into the S_3 supernatant fraction. For this study, the sonication times were varied from 1 to 15 min and the activities found in the R_3 and S_3 fractions are shown in Table 2. The highest specific activity (0.059 umoles Pi liberated/min/mg Pr) for ATPase activity was found in the R_3 fraction prepared by sonicating whole cells for 9-10 min. This was the sonication time used routinely in preparing the Azotobacter R_3 electron transport fraction. Total activity units recovered in the R_3 fraction are also greatest for this 9-10 min sonication time interval. The specific activities for ATPase activity in the S_3 fraction ranged from

TABLE 1

Distribution of Activity Units for ATPase in Fractions Obtained by Differential Centrifugation from Sonically Disrupted Resting Cells of Azotobacter vinelandii

Fraction	Protein Recovery (%)	Specific Activity[a]	Activity Total Units[b]	Activity Recovery (%)
S_1	100	0.031	49	100
S_2	92	0.025	36	73
R_2	9	0.028	4	8
S_2	100	0.025	36	100
S_3	63	0.004	4	11
R_3	24	0.052	18	50

[a]Expressed as umoles Pi liberated/min/mg protein at 30°.

[b]Total activity units were calculated by multiplying total protein concentration by specific activity.

TABLE 2

Effect of Time of Sonication on

Distribution of Protein and ATPase Activity

Time (min)	Protein Recovered (% of S_2 fraction)		ATPase Activity: Specific Activity [a]		ATPase Activity: Activity Recovered (% of S_2 fraction)	
	R_3	S_3	R_3	S_3	R_3	S_3
1	-	-	.005	.014	3.1	25.7
3	9.2	61.8	.040	.031	8.9	46.1
5	11.7	75.5	.054	.030	14.7	53.2
7	19.8	57.7	.056	.033	27.0	45.9
9	21.2	63.9	.059	.015	52.0	39.9
11	26.8	72.6	.056	.013	52.0	32.5
13	26.8	76.8	.056	.014	47.1	33.6
15	25.5	68.3	.028	.014	26.8	34.7

[a] Specific activity expressed as umoles Pi liberated per min per mg protein at 30°C.

0.014-0.033 and never became as high as the value found for the R_3 fraction. Increasing the time of sonication of whole cells past the 10 min time interval does not result in activity being released into the S_3 supernatant fraction although by increasing the sonication time interval some inactivation occurred as evidenced by a 50% loss in specific activity in the R_3 fraction prepared from cells after a 15 min sonication time. This does not represent heat denaturation since precautions were taken to keep all temperatures no higher than 10° during all exposures to sonication procedures. Therefore, all additional characterization studies for ATPase activity in the R_3 fraction were carried out using an R_3 electron transport fraction prepared after only a 10 min sonication interval.

Kinetic Studies on the Azotobacter ATPase Activity

Effect of reaction pH. The effect of reaction pH on ATPase activity is shown in Figure 2. Three buffer systems were utilized in order to obtain experimentally the desired pH range. Optimal ATPase activity occurs at pH 8.0 with considerable activity also occurring over the range pH 8.0-10.0.

Activation by Mg^{++} ion. The effect of varying concentrations of ATP in the absence and presence of different concentrations of Mg^{++} ion is shown in Figure 3. Maximal ATPase activity occurs

Figure 2. Effect of reaction pH on ATP hydrolysis by the Azotobacter R_3 fraction.

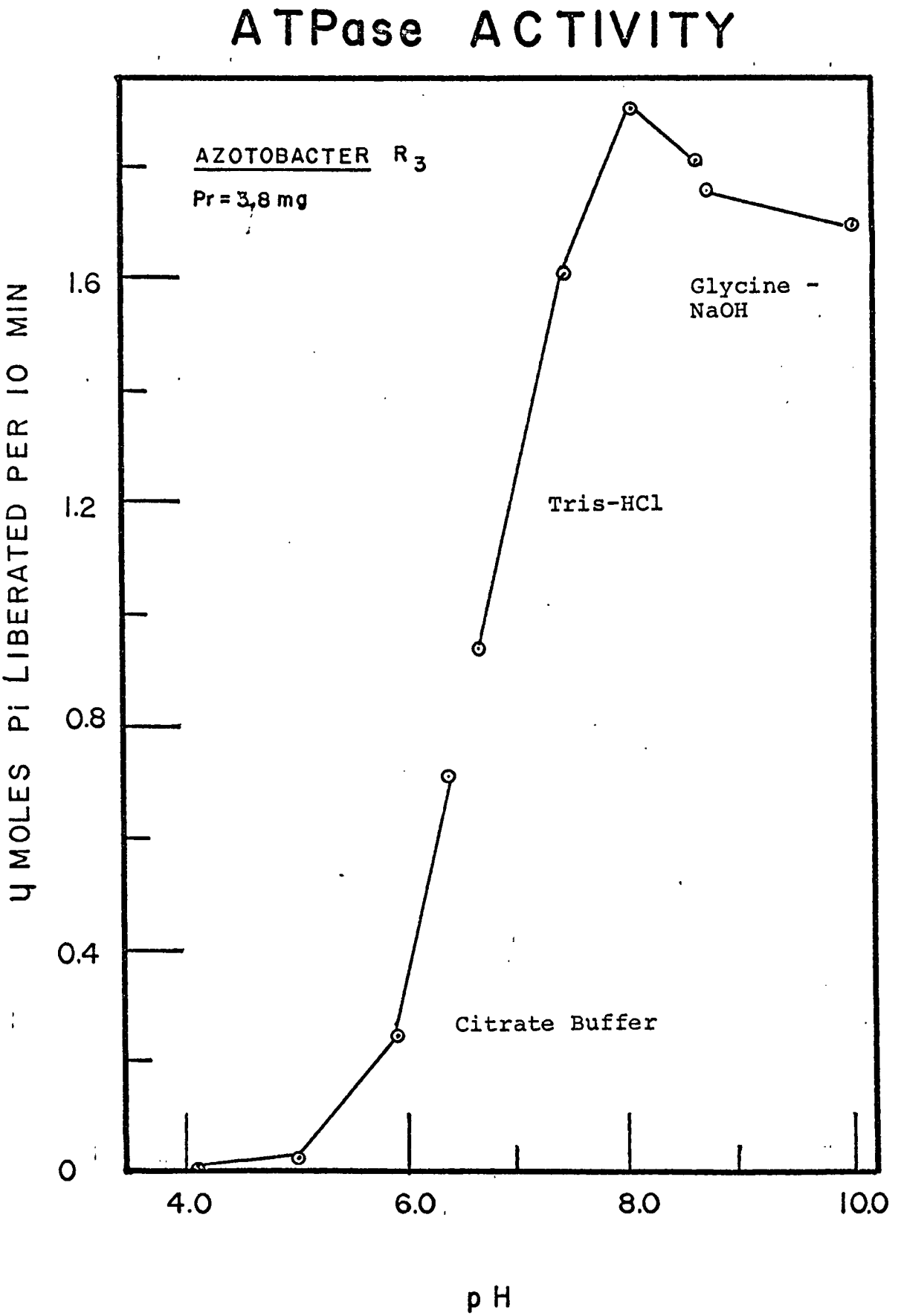
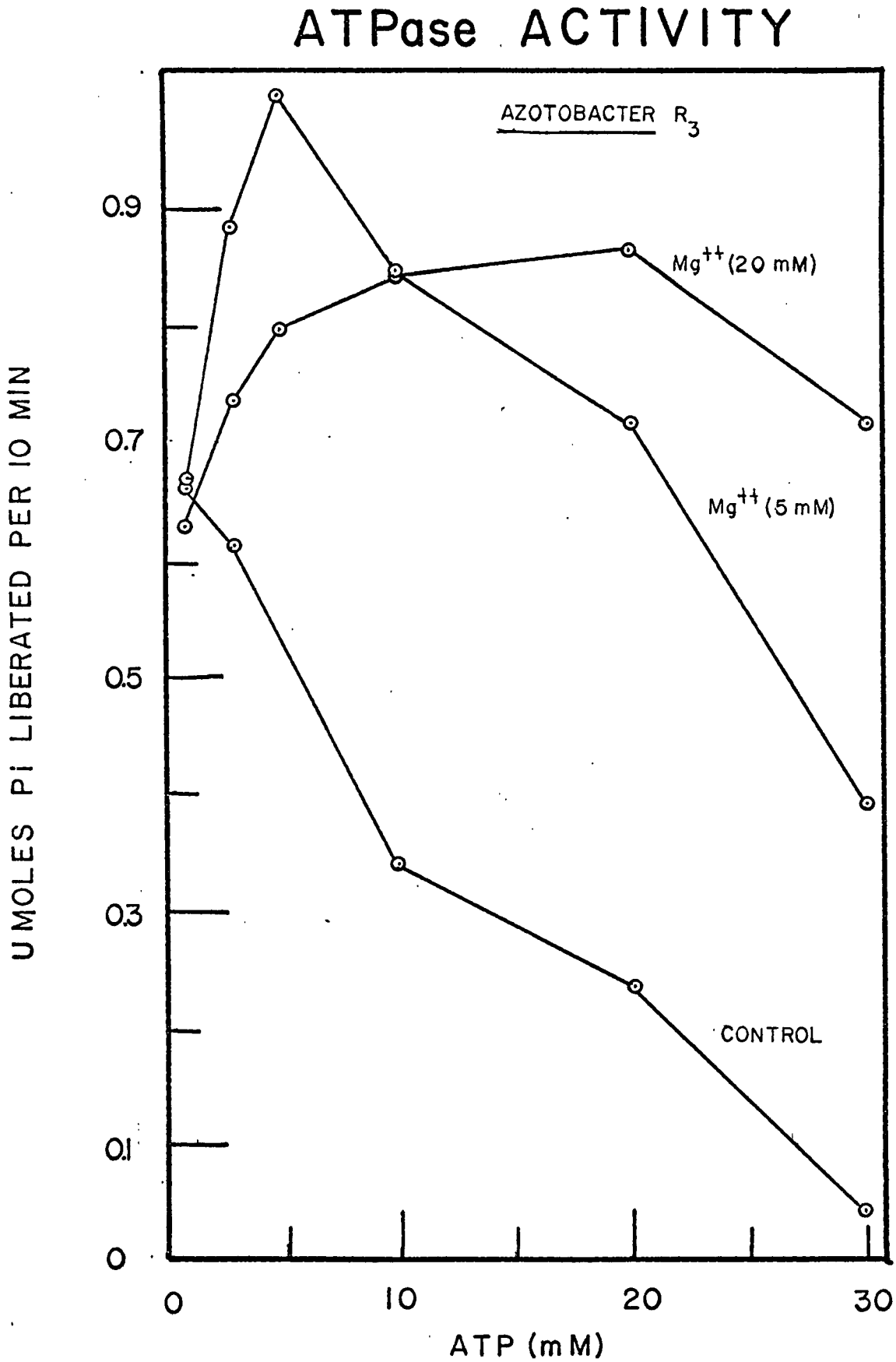

ATPase ACTIVITY
AZOTOBACTER R3
Pr = 3,8 mg
Glycine - NaOH
Tris-HCl
Citrate Buffer
μ MOLES Pi LIBERATED PER 10 MIN
0
0.4
0.8
1.2
1.6
4.0
6.0
8.0
10.0
pH

Figure 3. Effect of Mg^{++} ion concentration on ATP hydrolysis by the Azotobacter R_3 fraction at varying ATP concentration.


ATPase ACTIVITY
AZOTOBACTER R3
Mg++ (20 mM)
Mg++ (5 mM)
CONTROL
UMOLES Pi LIBERATED PER 10 MIN
0.9
0.7
0.5
0.3
0.1
0
0
10
20
30
ATP (mM)

at the point where both ATP and Mg^{++} ion were added at a 5 mM concentration level to the assay system. Another high activity point is noted where both ATP and Mg^{++} were added in equimolar concentrations at the 20 mM level. The control, which contained no externally added Mg^{++} ion shows a decrease in activity with increasing ATP concentrations, that is similar to those observed for the other curves where ATP concentration exceeds Mg^{++} ion concentration. The initial high activity for the control is probably due to residual Mg^{++} ion present in the Azotobacter R_3 fraction. The 1:1 molar ratio (of Mg^{++}:ATP) probably indicates the optimal substrate to metal ion concentration needed to form a Mg^{++}-ATP complex, the active substrate complex needed for enzyme turnover.

Effect of other cations. As shown by Table 3, Mn^{++} and Ca^{++} ions could replace Mg^{++} ion to some extent while all other cations tested were inhibitory.

Time course of reaction and stability. Figure 4 shows the kinetic study which defines the relationship of reaction time to ATPase activity. The two figures show the results of ATPase assays plotted as a function of two different time scales. ATPase activities for the various R_3 fractions are essentially linear with respect to the time intervals studied. The R_3

TABLE 3

Effect of Various Cations on ATPase Activity of the *Azotobacter* R_3 Fraction

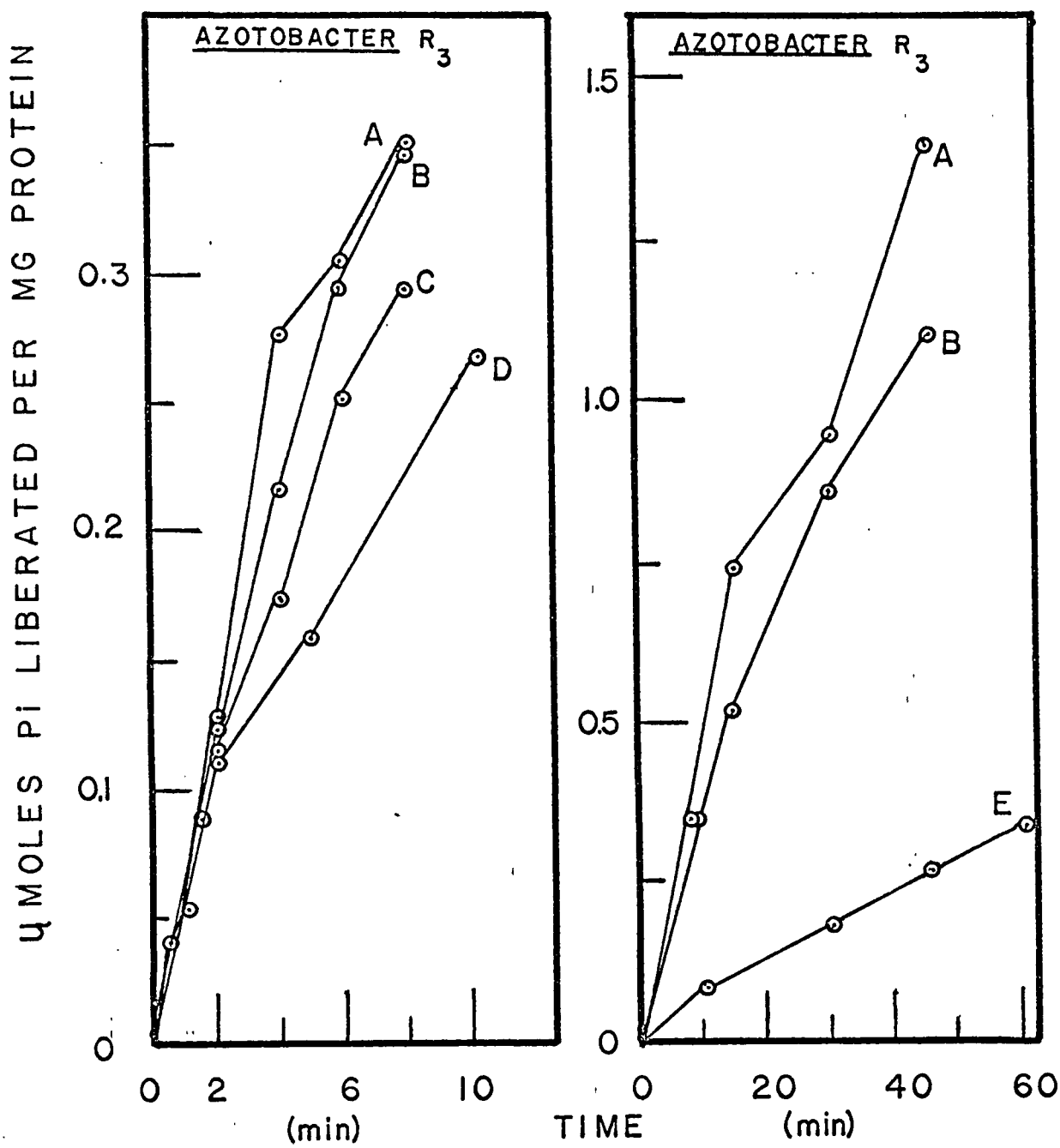
Cation[a]	S. A.[b]	Activity (%)
Mg^{++}	0.056	215
Mn^{++}	0.032	123
Ca^{++}	0.032	123
None	0.026	100
Co^{++}	0.013	50
$Na^{+} + K^{+}$	0.013	50
K^{+}	0.01	38
Na^{+}	0.01	38
Hg^{++}	0	0
Cu^{++}	0	0
Zn^{++}	0	0

[a]Final concentration of all metal cations was 5 mM.

[b]Specific activity expressed as umoles Pi liberated/mg/min at 30°.

Figure 4. Typical time course reactions for ATP hydrolysis by the Azotobacter R_3 fraction as plotted on two different time scales.

ATPase ACTIVITY



fraction designated A was prepared at temperatures of 15-25° to examine whether the Azotobacter enzyme was cold labile; B represents an R_3 fraction prepared from the same batch of resting cells but maintaining all preparatory temperatures at 4°. Curves C and D represent the activity of the same Azotobacter R_3 fraction as in curve A, only after overnight storage at room temperature (25°) and frozen (-20°), respectively. Curve E shows this same R_3 fraction after having been stored frozen (-20°) for two weeks. The similarity in activity patterns for both curves A and B show that the Azotobacter membrane-bound ATPase is not cold labile in the classical sense, since preparation at 4° causes no loss of activity. Overnight storage at room temperature results in a slight loss of activity, while overnight freezing results in a significant loss. By extending the freezing time interval greatly, one markedly inactivates ATPase activity. Frozen fractions (activity pattern of curve E) were resonicated and it was not possible to recover the original ATPase activity, suggesting that reaggregation of membranes by prolonged freezing was not responsible for this inactivation. Table 4 summarizes briefly the stability studies data which show conclusively that the temperature at which the Azotobacter R_3 fraction is prepared has little effect on the ATPase specific activity, but that the temperature and the duration of storage are important factors in maintaining high activity membrane fractions.

TABLE 4

ATPase STABILITY

AZOTOBACTER R_3

	S. A.[a]	%Loss
Room Temperature Prep.	.05	-
Cold Prep.	.05	-
Overnight Room Temp.	.043	.14
Overnight Refrigeration	.036	25
Overnight Frozen	.02	60
Frozen / Resonicated	.02	60
2 Days Frozen	.02	60

[a] Specific activity expressed as umoles Pi liberated per min per mg protein at 30°C.

Trypsin activation. Table 5 shows the effect of trypsin treatment on ATPase activity in the Azotobacter R_3 fraction. Treatment by trypsin markedly increases the specific activity and this increase ranged from 12-14 fold after trypsinization for 4 min at 25°. Since some bacterial ATPases require a binding protein for attachment to the membrane (10), which might be affected by proteases, a fractionation study was carried out with the Azotobacter R_3 fraction to determine whether or not trypsin treatment released the membrane-bound ATPase into the supernatant fraction. Figure 5 shows the results of this study and the data obtained. An R_3 fraction was trypsin treated as before but ultracentrifuged after adding trypsin inhibitor. The resulting residue (pellet) fractions were designated R_4 (heavy) which resembled the original R_3 fraction, and R_4 (light) which was a membrane fluff layer which overlayed the R_4 (heavy) fraction. The supernatant fraction designated S_4, represented those proteins that might have been solubilized by the trypsin treatment. The bulk of the ATPase activity remained membrane bound as evidenced by specific activities of 0.85 and 0.83 found in the R_4 (heavy) and R_4 (light) fractions as compared to the low activity of 0.17 found for the S_4 fraction. Furthermore, approximately 90 of the original 110 total activity units were recovered in the two R_4 particulate fractions while

TABLE 5

Stimulatory Effect of Trypsin on ATPase Activity in the AVO R_3 Fraction

System	Specific Activity[a]
Untreated R_3	0.024
Trypsin + R_3[b]	0.327
Inhibited trypsin + R_3[c]	0.016

[a]Specific activity expressed as umoles Pi liberated per min per mg protein at 30°.

[b]R_3 treated with trypsin (0.5 mg/mg protein) for 4 min at room temperature (25°).

[c]R_3 treated with inactivated trypsin.

Figure 5. Scheme outlining the fractionation of the trypsin-activated ATPase in the Azotobacter R_3 fraction and distribution of protein and ATPase activity units among the soluble and particulate fractions.

A. VINELANDII ATPase FRACTIONATION STUDY OF TRYPSIN ACTIVATION

	S.A.[a]	Pr (TOTAL)	UNITS (TOTAL)
R_3	0.06	150	9.0
TRYPSIN (0.5 mg/mg Pr)			
$Tr\text{-}R_3$	0.74	150[b]	110.9
144,000 x g - 120 MIN			
R_4 (HEAVY)	0.85	30.5	25.9
R_4 (LIGHT)	0.83	78	64.7
S_4	0.17	18.6[b]	3.1

[a] SPECIFIC ACTIVITY (u moles Pi/min/mg at 30°)

[b] EXCLUDING TRYPSIN PROTEIN

only three units were found in the S_4 supernatant fraction. The activation caused by trypsin treatment shows that the membrane fraction is in some way altered to allow for high turnover rates, and this may involve some type of membrane conformational change.

Inhibitor Studies. As shown in Table 6, no inhibition or stimulation resulted from addition of azide, 2,4-dinitrophenol, oligomycin, or ouabain to the standard reaction mixture.

TABLE 6

Effects of Various Compounds on the

ATPase Activity in the *Azotobacter* R_3 Fraction

Compound[a]	S. A.[b]	Inhibition (-) or Stimulation (+) (%)
Azide	0.05	0
Ouabain	0.05	0
Oligomycin	0.05	0
2,4-dinitrophenol	0.05	0

[a] All compounds were added to the standard assay system at a final concentration of 10^{-4} M, except oligomycin, of which 5 micrograms were added.

[b] Specific activity expressed as umoles Pi liberated per min per mg protein at 30°.

DISCUSSION

It should be apparent that many similarities exist among the various bacterial ATPases studied to date. They all have in common a requirement for divalent cations, either Ca^{++} or Mg^{++}, and maximal activation occurs at a molar ratio of cation:ATP that ranges from 0.5 - 1.0. They are all intimately associated with cytoplasmic membranes but are usually solubilized by relatively mild procedures, like prolonged washing with buffers. They many require activation by proteases (or heat) in order to unmask "latent" activity, and in many cases allotopic effects are observed between bound and soluble states. The ATPases when solubilized, are often inactivated by exposure to cold temperature (4°). Their response to inhibitors is variable and functional significance is largely speculative though various ATPases are thought to function in ion transport, nitrogen fixation, ATP-dependent reactions, and as coupling factors in phosphorylation systems. Those ATPases purified thus far have molecular weights of approximately 350,000 and usually are made up of subunits. A summary of many of the more interesting properties of bacterial ATPases is presented in Table 7.

TABLE 7

COMPARISON OF VARIOUS BACTERIAL ATPases

Characteristic	*S. faecalis*	*E. coli*	*E. coli*
Preparation	Soluble via washed protoplasts	Soluble via washed spheroplasts (no EDTA)	Soluble via wash of sonic memb.; DEAE
Molecular Data	MW=385,000, S_w=13.4S Subunits: 6 pairs	MW=365,000 - 390,000	MW 400,000
Stability & Latency	Bound stable, Soluble cold-labile	Bound stable, Soluble cold-labile 20% glycerol reduces lability	Bound stable, soluble cold-labile; 20% MeOH or 50% glycerol protects
Cation Activation	Mg^{++} required Mg^{++}:ATP = 1.0 $Na^+ + K^+$, no effect	Mg^{++} or Ca^{++} required Mg^{++}:ATP=0.4 Ca^{++}:ATP=0.66 Na^++K^+ inhibitory	Mg^{++} required; Mg^{++}:ATP=0.4; Na^+ slight activate Na^++K^+ inhibit.
pH	8.0	8.5 - 9.5	9.5
Inhibitors	ADP, Pi, DCCD, Dio-9, guanidines	Li^+, Cs^+ ADP inhib. Ca^{++} ATPase	ADP, azide, DCCD, guanadine, DNP, ouabain, KCN, oligomycin no effect
Other	10 nm hexagon, no stalks; no phosphorylated intermediates or exchange	ADP hydrolyzed by Mg^{++} ATPase E*= 20.7 kcal/mole	—
Author & Reference	Abrams, Baron, Schnebli Smith, (1-6,8,10,34,67,68)	Davies, Bragg (15)	Kobayashi, Anraku (41)

Table 7 (continued)

Characteristic	*E. coli*	*E. coli*	*B. megaterium* KM
Preparation	Membranes	Sonic, Triton-X, DEAE, purified	Purified from soluble via washed ghosts, DEAE
Molecular Data	-	MW=360,000 S_w=12.9 Subunits MW: 60,000 56,000 35,000 13,000	MW=375,000-410,000 Subunit MW: 68,000 65,000
Stability & Latency	Heat-activated	Cold labile, breaking down into subunits	Bound and soluble slowly inact. at 4°
Cation Activation	Mg^{++}:ATP = 0.4 Ca^{++}:ATP = 0.2 Depends on pH	Mg^{++} or Ca^{++} slightly by Co^{++}, Ni^{++}, Mn^{++}	Ca^{++} Mg^{++} Ca^{++}:ATP =1:1-1:5 K^+, Na^+ no effect
pH	9.0 for Mg^{++} 9.9 for Ca^{++}	7.8	7.2-7.8
Inhibitors	Azide, PCMB, PCP Ca^{++}-ATPase inhib by Na^+, K^+, Li^+, Rb^+, Cs^+	Azide non-comp. Pi " " KCN no effect	ADP competitive
Other	One enzyme activated by Ca^{++} or Mg^{++} depending on pH; possible regulatory involvement.	Antibody inhibits ATPase & all related activity. No sigmoidal kinetics.	On inner membrane with some exposure to outer.
Author & Reference	Evans, (19)	Hanson & Kennedy (31)	Mirsky & Barlow (46-49)

Many of the findings for the Azotobacter ATPase presented here, are consistent with properties described for other bacterial ATPases. It should be emphasized that this study represents only a preliminary characterization of the membrane-bound ATPase of Azotobacter vinelandii, the results of which are a prerequisite for further studies on the isolation, purification and characterization of the ATPase in this bioenergetically diverse organism. Although some of the variables defined in this study were used in previous work, this study represents the first attempt to determine conditions for optimal hydrolysis of ATP by the membrane-bound ATPase and attempts to show its association with cytoplasmic membrane, especially that membrane fraction known to contain the highest concentration of electron transport enzymes. As stated in the results, the Azotobacter ATPase appears to be intimately associated with the membranous network that is very extensive in this organism. Preliminary indications are that Azotobacter ATPase is apparently more tightly bound to membranes than most bacterial ATPases since neither extended sonication or trypsin treatment caused its release into the soluble or supernatant fraction. This close association of this enzyme with the membrane-bound electron transport system, its trypsin activation and other properties, as well as its ability to serve as a coupling factor in oxidative phosphorylation (17) leaves little doubt that the ATPase of

Azotobacter vinelandii, though possibly multifunctional, is at least involved in coupling and energy conservation. The multitude of energy requiring processes in Azotobacter warrant purification and further study of the ATPase to establish its relationship to structural, physiological and bioenergetic processes in the organism. In such a continuation study, the information obtained by this investigation will be required.

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