Journal of Supramolecular Structure 3:304–313 (1975)

MAGNETIC RESONANCE AND KINETIC STUDIES OF THE MECHANISM OF MEMBRANE-BOUND SODIUM AND POTASSIUM ION-ACTIVATED ADENOSINE TRIPHOSPHATASE

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EPR and water proton relaxation rate ($1/T_1$) studies of partially (40%) and "fully" (90%) purified preparations of membrane-bound ($Na^+ + K^+$) activated ATPase from sheep kidney indicate one tight binding site for Mn^{2+} per enzyme dimer, with a dissociation constant ($K_D = 0.88\ \mu M$) in agreement with the kinetically determined activator constant, identifying this Mn^{2+}-binding site as the active site of the ATPase. Competition studies indicate that Mg^{2+} binds at this site with a dissociation constant of 1 mM in agreement with its activator constant.

Inorganic phosphate and methylphosphonate bind to the enzyme-Mn^{2+} complex with similar high affinities and decrease $1/T_1$ of water protons due to a decrease from four to three in the number of rapidly exchanging water protons in the coordination sphere of enzyme-bound Mn^{2+}. The relative effectiveness of Na^+ and K^+ in facilitating ternary complex formation with HPO_4^{2-} and $CH_3PO_3^{2-}$ as a function of pH indicates that Na^+ induces the phosphate monoanion to interact with enzyme-bound Mn^{2+}, while K^+ causes the phosphate dianion to interact with the enzyme-bound Mn^{2+}. Thus protonation of an enzyme-bound phosphoryl group would convert a K^+-binding site to a Na^+-binding site. Dissociation constants for K^+ and Na^+, estimated from NMR titrations, agreed with kinetically determined activator constants of these ions consistent with binding to the active site.

Parallel $^{32}P_i$-binding studies show negligible formation ($< 7\%$) of a covalent E-P complex under these conditions, indicating that the NMR method has detected an additional noncovalent intermediate in ion transport. Ouabain, which increases the extent of phosphorylation of the enzyme to 24% at pH 7.5 and to 106% at pH 6.1, produced further decreases in $1/T_1$ of water protons. Preliminary ^{31}P-relaxation studies of $CH_3PO_3^{2-}$ in the presence of ATPase and Mn^{2+} yield an Mn to P distance (6.9 ± 0.5 Å) suggesting a second sphere enzyme-Mn-ligand-$CH_3PO_3^{2-}$ complex.

Previous kinetic studies have shown that Tl^+ substitutes for K^+ in the activation of the enzyme but competes with Na^+ at higher levels. From the paramagnetic effect of Mn^{2+} at the active site on the enzyme on $1/T_1$ of ^{205}Tl bound at the Na^+ site, a Mn^{2+} to Tl^+ distance of 4.0 ± 0.1 Å is calculated, suggesting the sharing of a common ligand atom by Mn^{2+} and Tl^+ on the ATPase. Addition of P_i increases this distance to 5.4 Å consistent with the insertion of P between Mn^{2+} and Tl^+.

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These results are consistent with a mechanism for the $(Na^+ + K^+)$-ATPase and for ion transport in which the ionization state of P_i at a single enzyme active site controls the binding and transport of Na^+ and K^+, and indicate that the transport site for monovalent cations is very near the catalytic site of the ATPase. Our mechanism also accounts for the order of magnitude weaker binding of Na^+ compared to K^+.

INTRODUCTION

The sodium and potassium ion-activated adenosine triphosphatase plays an important role in directing metabolic energy to the active transport of sodium and potassium ions across the membranes of many animal cells (1, 2). Although numerous studies have been carried out on the $(Na^+ + K^+)$-ATPase, the detailed mechanism for catalysis has not been established. We have been using a variety of magnetic resonance techniques to probe the active site of the ATPase and to study its association with the transport sites for Na^+ and K^+ (3, 4). From EPR and water proton relaxation rate (PRR) studies of the partially (40%) purified enzyme, a single tight Mn^{2+} binding site was found. The dissociation constants of Mn^{2+} (0.88 μM) and Mg (1 mM) from this tight binding site agree with their respective activator constants for ATP hydrolysis determined kinetically, suggesting that this tight binding site represents the catalytically active site of the enzyme. A large number of much weaker Mn^{2+} binding sites stoichiometric with the phospholipid phosphorus were also detected, suggesting binding sites on the lipid membrane as well (3).

The interactions of the partially purified enzyme with inorganic phosphate and a phosphate analog, methylphosphonate, have been examined in a series of PRR studies. Phosphate and methylphosphonate decrease the enhancement of water proton relaxation by the ATPase by 26% and 24%, respectively, in the presence of both Na^+ and K^+ at pH 7.5, suggesting the formation of ternary enzyme-Mn^{2+}-substrate complexes. A study of the individual effects of Na^+ and K^+ as a function of pH indicated that the presence of Na^+ is necessary for the phosphate monoanion to form a ternary enzyme-Mn^{2+}-phosphate complex, while K^+ is required for the phosphate dianion to form a ternary complex (3). Thus, protonation of an enzyme-bound phosphoryl group could convert a K^+-binding site to a Na^+-binding site, increasing the stoichiometry of Na^+ binding from zero to one, and decreasing the stoichiometry of K^+ binding from one to zero without changing the measured dissociation constant of either cation.

Dissociation constants for Na^+ and K^+ estimated from our nuclear magnetic resonance titrations agreed with kinetically determined activator constants of these ions, consistent with binding to the active site. A mechanism for the ATPase and for ion transport was proposed, on the basis of these observations (3). Several aspects of this mechanism have since been tested. Thus, the predicted propinquity between the monovalent and divalent cation sites has recently been established by ^{205}Tl-NMR (4).

The present paper describes additional tests of our mechanism. We have modified the procedure of Jorgensen (5) to obtain a highly purified ATPase preparation which we judge by SDS gel electrophoresis to be 91% pure. The existence of the single tight Mn^{2+} binding site on the ATPase was re-examined in this preparation in an EPR study. The high affinity of the ATPase-Mn^{2+} complex for phosphate and methylphosphonate (3) raised the question of whether the NMR method was also detecting the well-known covalent phosphoenzyme complex (6, 7). This point was examined by $^{32}PO_4H^{2-}$ binding studies. A

study of the frequency dependence of $1/T_1$ of water had indicated that the binding of phosphate or methylphosphonate resulted in a decrease from four to three in the number of rapidly exchanging water protons in the coordination sphere of enzyme-bound Mn^{2+} (3). Such effects could be due either to the displacement or occlusion of a coordinated hydroxyl ion by phosphate or methyl phosphonate. This point was investigated in a ^{31}P-NMR study of the interactions of $CH_3PO_3^{2-}$ with the ATPase-Mn^{2+} complex.

MATERIALS AND METHODS

Two preparations were used in these studies: a partially purified preparation, estimated by specific activity and SDS-gel electrophoresis (8) to be 40% pure, and a purified enzyme, which is more than 90% pure on the basis of the same criteria. The preparation of the 40% enzyme has been described previously (9). Our 91% pure enzyme was prepared with a modification of Jorgensen's method (5). Microsomes were isolated as described by Schwartz et al. (10). Jorgensen's rapid purification procedure which uses a discontinuous sucrose gradient in an angle rotor was followed exactly. The specific activity at this stage was typically 17–19 μmoles ATP hydrolyzed per mg of protein per min. This material was then treated a second time with SDS in the following incubation mixture: 3 mM NaATP, 2 mM EDTA, 0.58 mg SDS/ml, 1.4 mg protein/ml, 50 mM imidazole, pH 7.5 (20°C). After a 30-min incubation at 20°C, the sample was layered onto 35 ml sucrose gradients which were 16.5–40.0% (wt/wt) and centrifuged at 27,000 rpm (95,000 × g in a Beckman SW27 rotor for 4 hr at 0°–4°C. Fractions were obtained from the gradients from a puncture in the bottom of the tube. The fractions containing the peak enzyme activity were pooled, diluted threefold with distilled water, and centrifuged at 36,000 rpm for 2 hr in a Beckman 60 Ti rotor. The pellets were resuspended in 10% sucrose to a protein concentration of 2 mg/ml and immediately frozen at −20°C. Activity of the final preparation was typically 37–39 μmoles ATP hydrolyzed per mg of protein per min.

The extent of phosphorylation of the enzyme was determined after incubation at 25°C of the partially purified enzyme (0.1 μM tight Mn-binding sites) with 20 μM ^{32}P-labeled phosphate in the presence of 25 mM TMA-PIPES buffer (pH 6.1) or 25 mM TMA-TES buffer (pH 7.5), TMA Cl to adjust the ionic strength to 110 mM, 10 mM $MgCl_2$, or 10 μM $MnCl_2$, as well as 10 mM KCl or 100 mM NaCl in a total volume of 0.5 ml. The reaction mixture was terminated after 1 min by the addition of 3 ml of 10% trichloroacetic acid containing 10^{-4} M TMA phosphate. The mixture was centrifuged at 7,000 × g for 20 min. The pellet was washed five times with the cold (2°C) trichloroacetic acid-phosphate mixture. The pellet was then dissolved in 0.1 N NaOH and an appropriate aliquot was added to Hydromix scintillation fluid (New England Nuclear, Boston, Mass.) for counting.

EPR spectra and pulsed NMR measurements of water proton relaxation rates were made as previously described (3). The ^{31}P-relaxation rates were determined by pulsed Fourier transform NMR with proton decoupling as previously described (11). Sample volumes of 0.9 ml were placed in a 10-mm NMR tube. The 10-mm NMR tube was inserted into a concentric 12-mm NMR tube which contained D_2O for field-frequency locking, avoiding direct contact between the ATPase and inhibitory D_2O.

RESULTS

Properties of the Highly Purified Enzyme

The enzyme purification procedure described here yields material of high specific activity (37–39 U/mg) and appears to be 91% pure on the basis of SDS gel electrophoresis. As shown in Fig. 1 the gels show two major bands and one minor contaminant. The molecular weights of the major bands are 90,000 and 55,000, in reasonable agreement with the data presented on several other preparations (12–14). Scans of the gels at 600 nm indicate an optical density ratio of 1.66 for the large peptide over the small peptide, yielding a molar ratio of 1.02 ± 0.10 mole of large peptide per mole of small peptide. Jorgensen (12) and Hokin (13) have reported finding a ratio of 2 moles of large peptide per mole of small peptide, whereas Kyte (14) has obtained a preparation with one large peptide per two small peptides. These differences result mainly from differences in the relative yields and assumed corrections to the molecular weights of the large and small peptides.

A Mn^{2+} binding study was carried out on the 91% pure enzyme and the results are shown in Fig. 2. As can be seen, no free Mn^{2+} was detected until the ratio of Mn^{2+} to enzyme became greater than one. From an estimate of the maximum amount of free Mn^{2+} which could have been observed under these conditions, an upper limit for the dissociation constant, K_D, of 1.0 μM was obtained, in good agreement with the previously determined value of 0.88 μM, determined both from proton relaxation studies and kinetic studies of the partially purified enzyme (3).

^{32}P and PRR Studies of Covalent Phosphate Binding

In order to examine the covalent phosphoenzyme complex, which has been proposed to be an intermediate in the ATPase reaction (6, 7), a series of ^{32}P phosphate-binding studies were carried out. As shown in Table I, in the absence of ouabain, at both pH 6.1 and 7.5, a very low level of phosphorylation ($\leqslant 0.07$ moles $^{32}PO_4$ incorporated per mole of enzyme) was observed with Mn^{2+}, indicating that the complex previously detected by the PRR studies is not the covalent E-P complex. With Mg^{2+} alone or in the presence of K^+, significant phosphorylation was detected at pH 6.1 but not at 7.5. Since Mg^{2+} is not paramagnetic, these effects could not be further studied by PRR.

If the complex observed in the PRR titrations with Mn^{2+} had been the covalent E-P intermediate, one would expect that Na^+ should promote phosphorylation at pH 6.1 and that K^+ should promote phosphorylation at pH 7.5 This was not observed in the absence of ouabain. However, when ouabain was present, a greater phosphorylation was observed with Na^+ than with K^+ at low pH, and a greater phosphorylation was seen with K^+ than with Na^+ at high pH.

The greatest effect of ouabain on a paramagnetic complex occurred at pH 6.1 in the presence of Mn^{2+}, or with Mn^{2+} plus Na^+. PRR titrations under similar conditions (Table I) show that phosphate causes 4–35% greater decreases in the enhancement of water proton relaxation in the presence of ouabain than in its absence. The lack of a simple correlation between the extent of phosphorylation and the decrease in the enhancement of water proton relaxation indicates that the NMR method is detecting a non-covalent phosphate complex of the ATPase.

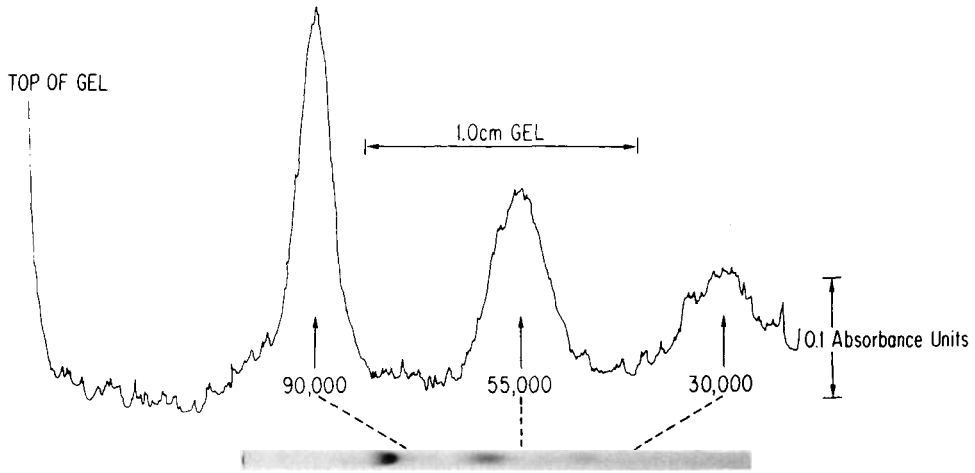

Fig. 1. Spectrophotometric scan at 600 nm of highly purified ($Na^+ + K^+$)-ATPase. SDS gels were stained with amido-black. The indicated molecular weights of the components were determined by calibration of the SDS gels with phosphorylase B (92,500), bovine serum albumin (64,000), ovalbumin (43,000), and myoglobin (17,500). The peak on the right of molecular weight 30,000 is probably a contaminant representing 9% of the integrated area. The ATPase components (90,000 and 55,000) are present in a 1.02:1.00 mole ratio.

^{31}P-NMR Studies of the ATPase-Mn^{2+}-$CH_3PO_3^{2-}$ Complex

Our previous studies of the binding of Mn^{2+}, monovalent cations and substrates indicated that the Mn^{2+} and phosphate or methylphosphonate binding sites might be very near each other on the ATPase. If this were the case, one would expect the relaxation rates of the ^{31}P nucleus of P_i or methylphosphonate to be increased upon interaction with the enzyme-Mn^{2+} complex, due to electron-nuclear dipolar interactions. In the present studies, we chose methylphosphonate due to its greater dissociation constant from its ternary complex (7.3 μM) than that of phosphate (4.8 μM). Table II shows the effect of Mn^{2+} on the longitudinal ($1/T_1$) and transverse ($1/T_2$) relaxation rates of the methylphosphonate phosphorus in the presence of the enzyme. From Table II, the increases in the relaxation rates are proportional to the Mn^{2+} concentration. Moreover, since the ratio of the normalized relaxation rates ($1/fT_{2_p}:1/fT_{1_p} = 26$) is significantly greater than one, exchange contributions to $1/fT_{1_p}$ can be ruled out, and $1/fT_{1_p}$ can be used to calculate the Mn^{2+} to phosphorus distance. Addition of excess P_i in an amount calculated to displace 82% of the $Ch_3PO_3^{2-}$ caused a 90% decrease in both $1/fT_{1_p}$ and $1/fT_{2_p}$ consistent with such displacement. From the previously described frequency dependence of the water proton relaxation rate (3), a correlation time, τ_c of 1.88×10^{-9} sec for the Mn^{2+}-H_2O dipolar interaction was obtained in the same complex. Since this value is almost certainly dominated by the electron spin relaxation time of Mn^{2+}, the τ_c for the methylphosphonate interaction is probably also the electron spin relaxation time. By using this value for τ_c and the values of $1/fT_{1_p}$ from Table II, the Solomon-Bloembergen

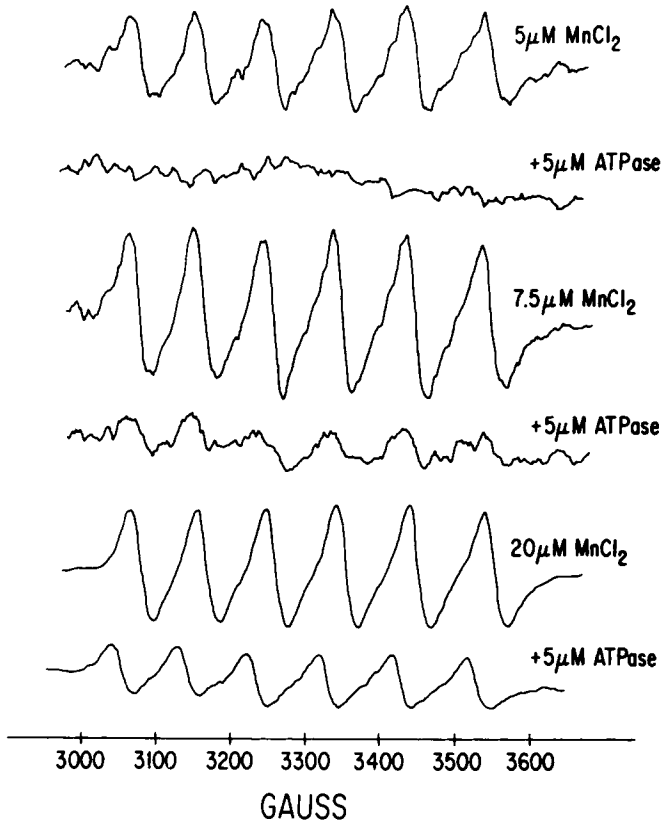

Fig. 2. Electron spin resonance spectra of Mn^{2+} in the absence and presence of 5 μM highly purified $(Na^+ + K^+)$-ATPase. All samples contained 25 mM TMA-TES, pH 7.5, 100 mM NaCl, and 10 mM KCl. Spectra were made at 22°C using a scan rate of 500 Gauss/min and a time constant of 0.3 sec. Eight scans of each sample were collected in a Nicolet-1074 instrument computer and read out at relative gains of 1.00 (upper and middle pair) and 0.25 (lower pair).

equation (15, 16) was used to calculate a distance between Mn^{2+} and the phosphorus of methylphosphonate on the ATPase of 6.9 ± 0.5 Å. This distance is too great for direct coordination of $CH_3PO_3^{2-}$ by the enzyme-bound Mn^{2+} which would yield a distance of 3.2 Å (2.8–3.8 Å) (17, 18), but is appropriate for a second-sphere enzyme-Mn-L-$CH_3PO_3^{2-}$ complex in which a ligand (L) intervenes between the enzyme-bound Mn^{2+} and $CH_3PO_3^{2-}$. The nature of the ligand, L, is not directly determined, but from the PRR studies (3) and from the data of Table II, it could be either a slowly exchanging hydroxyl ion (Fig. 3) or some other slowly exchanging ligand, such as a coordinated molecule of $CH_3PO_3^{2-}$.

From the $1/fT_{2_p}$ value (Table II), a lower limit to the rate of dissociation of $CH_3PO_3^{2-}$ from its enzyme complex ($k_{off} \geqslant 5.1 \times 10^4$ sec^{-1}) is obtained. From this value and the dissociation constant of 7.3 μM, a second order rate constant ($k_{on} \geq 7.0 \times 10^9$ M^{-1} sec^{-1}) is obtained which indicates diffusion-controlled formation of the second sphere ATPase-Mn-L-$CH_3PO_3^{2-}$ complex.

TABLE I. ^{32} Phosphate Binding and PRR Studies with the Partially Purified ATPase[1]

pH	[NaCl] mM	[KCl] mM	[Ouabain] mM	Moles ^{32}P bound/mole ATPase Mg^{2+}	Moles ^{32}P bound/mole ATPase Mn^{2+}	$(\epsilon_T/\epsilon_b)^2$
6.1	–	–	–	1.26	0.07	1.00
	100	–	–	0.13	0.04	0.87
	–	10	–	0.55	0.07	–
	–	–	0.1	1.10	1.17	0.65[3]
	100	–	0.1	1.02	0.96	0.83[3]
	–	10	0.1	0.64	0.13	–
7.5	–	–	–	0.10	0.06	0.79
	100	–	–	0.06	0.04	0.96[4]
	–	10	–	0.06	0.04	0.73[4]
	–	–	0.1	0.33	0.24	–
	100	–	0.1	0.08	0.07	–
	–	10	0.1	0.24	0.07	–

[1] Conditions are as described in Materials and Methods.
[2] (ϵ_T/ϵ_b) is the ratio of the enhancement of the water proton relaxation rate in the ternary ATPase-Mn^{2+}-P_i complex (ϵ_T) to that in the binary ATPase-Mn^{2+} complex (ϵ_b). $\epsilon_b = 8.0 \pm 1.0$ (3).
[3] Ouabain, when present in the PRR titration, was 25 μM.
[4] From reference 3.

TABLE II. ^{31} P Relaxation Studies of Methyl Phosphonate with the Partially Purified ATPase

	$1/T_1$ sec^{-1}	$1/fT_{1p}$ sec^{-1} $\times 10^{-3}$	$1/T_2$ sec^{-1}	$1/fT_{2p}$ sec^{-1} $\times 10^{-4}$
10 mM CH_3PO_3				
+ 4.4 μM (Na^+ + K^+)-ATPase	0.119	–	4.63	–
+ 0.55 μM $MnCl_2$	0.235	2.1	7.58	5.3
+ 1.1 μM $MnCl_2$	0.339	2.0	10.1	4.9
+ 2.2 μM $MnCl_2$	0.556	1.9	–	–
+29.4 mM TMA-PO_4	0.171	0.2	5.75	0.5

A solution containing 10 mM TMA-$CH_3PO_3^{2-}$, 4.4 μM ATPase sites, 25 mM TMA-TES (pH 7.5), 100 mM NaCl, and 10 mM KCl was titrated with Mn Cl_2, measuring $1/T_1$ and $1/T_2$ as indicated above. Temperature 23°C.
$1/fT_{1p}$ and $1/fT_{2p}$ are the paramagnetic contributions to the longitudinal and transverse relaxation rates respectively, normalized by the factor $f = [Mn^{2+}] / [CH_3PO_3^{2-}]$.
Conditions were otherwise as described in Materials and Methods. The error in $1/fT_{1p}$ is ±5% and in $1/fT_{2p}$ is ± 10%.

DISCUSSION

The Mn^{2+} binding data with the highly purified enzyme confirm our earlier studies of the partially purified enzyme (3) which indicated a single Mn^{2+} binding site per enzyme molecule, of molecular weight 250,000–300,000. Such a molecule appears to consist of two apparently identical large subunits of molecular weight 90,000 each, and two apparently identical small subunits of molecular weight 35,000–55,000. The Mn^{2+} binding studies thus yield a "half-site" stoichiometry.

By using this Mn^{2+} binding site as a paramagnetic reference point, structural information on several functional complexes has been obtained by EPR as well as by proton, ^{31}P, and ^{205}Tl relaxation studies. This information is summarized in Fig. 3, which shows reasonable structures for the binary enzyme-Mn complex and for two noncovalent quaternary complexes: the enzyme-Mn^{2+}-Na^+-phosphate monoanion complex and the enzyme-Mn^{2+}-K^+-phosphate dianion complex. The propinquity of Mn^{2+} to the monovalent cation has previously been established by ^{205}Tl NMR which revealed Mn^{2+} to Tl distances of 4.0 Å in the absence and 5.4 Å in the presence of P_i (4). The former distance suggests the sharing of a common ligand atom of the protein by Mn^{2+} and Tl^+, while the latter distance is consistent with the insertion of phosphate between the bound Mn^{2+} and bound Tl^+. Quantitative displacement experiments by K^+ and Na^+ indicated that the Tl^+ site under investigation was the Na^+ binding site of the ATPase (4).

The interaction of the enzyme-bound Mn^{2+} with methylphosphonate is shown in this paper to be a second-sphere complex rather than an inner sphere complex as we had previously suggested (3, 4). All of the measured dissociation constants of the cations from their respective complexes satisfy the rate equation of the ATPase, consistent with the view that they represent catalytically active complexes (3, 4).

The covalent E-P complex detected in the presence of ouabain (Table I) may well represent a later intermediate in ion transport. Of interest is the finding that the maximal amount of phosphorylation is closer to one per dimer rather than two, further suggesting a half-site stoichiometry.

A mechanism for the ATPase and for ion transport consistent with our data is shown in Fig. 4. This mechanism uses the covalently bound phosphoryl monoanion as a Na^+ carrier and phosphoryl dianion as a K^+ carrier, and predicts, therefore, that proton transport should accompany Na^+ transport (3). From the $1/fT_{2p}$ values (Table II), the second sphere ATPase-Mn-$CH_3PO_3^{2-}$ complex dissociates at a rate (5.1×10^4 sec^{-1}) much greater than the turnover number of the ATPase (23.1 sec^{-1}). Hence, the covalent phosphoenzyme complex, which does not easily dissociate phosphate, would be a more likely carrier for monovalent cations than the second sphere complex (Fig. 4). A mechanism of this type has been proposed by Mitchell for oxidative phosphorylation (19). The role of the hydrolysis of ATP is to provide a proton as well as the phosphoryl group, although other means of providing these components could contribute to ion transport.

Mobility of the monovalent ion carrier during Na^+ and K^+ transport appears to be low or absent. Thus, Kyte has shown immunologically that high amplitude motions of The ATPase molecule are unlikely (20), and Stoeckenius has provided laser Raman spectroscopic data in support of the sequential binding of protons to several sites in a membrane system during the light-induced transport of protons, requiring no mobile carriers at all (21). In a mechanism with little or no mobility of the Na^+ and K^+ carriers, the bound phosphoryl group (Fig. 4) could serve as one of several sites or way stations for monovalent cations during transport.

The subunit composition of the ATPase suggests a double dimeric structure and raises the possibility of site-to-site interactions. Such interactions are further supported by the half-site stoichiometries found for the binding of Mn^{2+} and P_i. Half-site stoichiometry could represent an extreme form of negative cooperativity between a pair of identical divalent cation binding sites. Negative cooperativity in

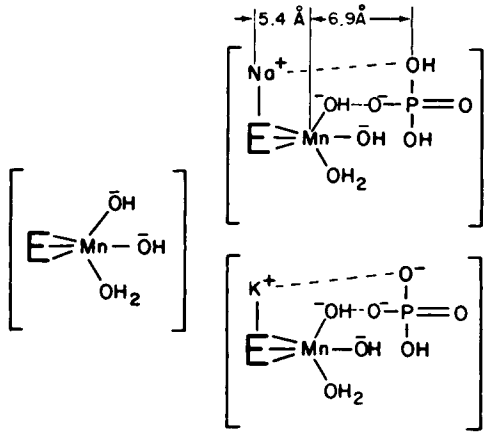

Fig. 3. Structures of ATPase complexes determined by electron and nuclear magnetic resonance. The Mn^{2+} stoichiometry was determined by EPR (Fig. 2 and Ref. 3). The number of protons in the coordination sphere of Mn^{2+} and the state of protonation of phosphate in the Na^+ and K^+ complexes were determined by titrations measuring water proton relaxation rates (3). The Mn^{2+} to Na^+ distance was determined by thallium relaxation rates (4) and the Mn^{2+} to phosphorus distance was determined by ^{31}P relaxation rates in the methyl phosphonate complex (Table II).

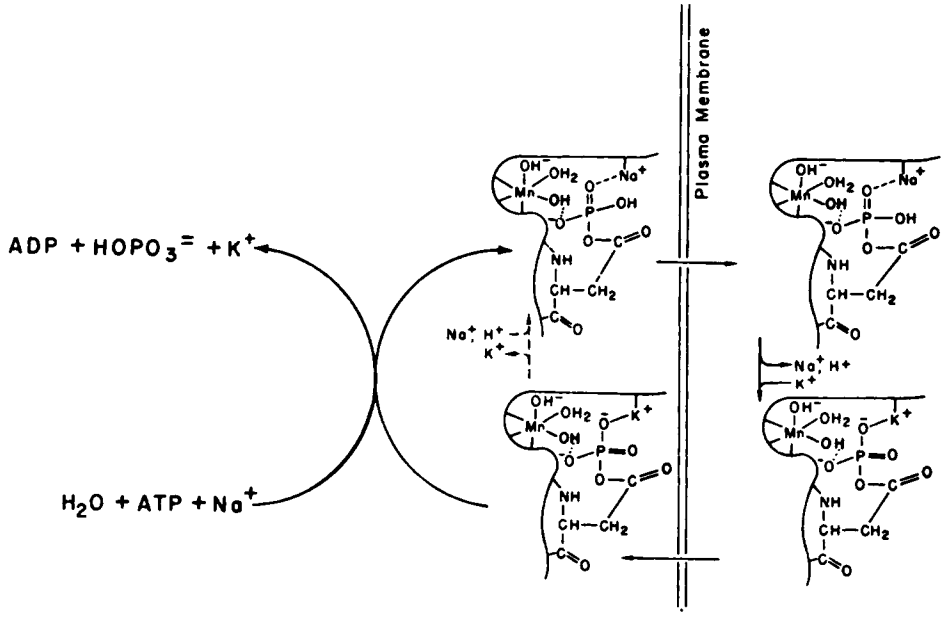

Fig. 4. Mechanism of the $(Na^+ + K^+)$-ATPase and monovalent cation transport suggested by the structures of Fig. 3. Hydrolysis of ATP or other reactions provide protons. Protonation of the enzyme-bound phosphoryl group ("inside" the cell) facilitates the binding of Na^+. Deprotonation of the phosphoryl group ("outside" the cell) releases Na^+ and facilitates K^+ binding.

an enzyme which traverses a membrane, such as the $(Na^+ + K^+)$ ATPase (22, 23), could provide a means of transferring information across the cell membrane. Thus, the reversible binding of a divalent cation to an external site of the $(Na^+ + K^+)$ ATPase would prevent the binding of Mg^{2+} to the internal site, reversibly inhibiting ATP hydrolysis and Na^+ transport.

ACKNOWLEDGMENTS

We are grateful to Dr. R. K. Gupta for help with the magnetic resonance instrumentation and to Dr. L. Cohen and Miss G. Moffitt for advice on gel electrophoresis.

This work was supported by National Institutes of Health grant AM-13351, National Science Foundation grant BO-41857, and by grants to this Institute from the National Institutes of Health (CA-06927 and RR-05539) and by an appropriation from the Commonwealth of Pennsylvania. C. M. Grisham was a Post Doctoral Fellow of the National Cancer Institute, NIH, in 1974.

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