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Calorimetric Studies of the Interaction of Magnesium and Phosphate with (Na^+,K^+)ATPase: Evidence for a Ligand-Induced Conformational Change in the Enzyme†

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ABSTRACT: The phosphorylation of (Na^+,K^+)ATPase from the electric organ of the electric eel is dependent on Mg^{2+}. The amount of phosphoenzyme formed was increased by K^+ and decreased by Na^+. Kinetic analyses indicate that a ternary complex of ATPase, P_i, and Mg^{2+} is formed prior to phosphorylation of the protein. Calorimetric studies revealed extraordinarily large enthalpy changes associated with the binding of Mg^{2+} (−49 kcal/mol) and of P_i (−42 kcal/mol), indicating a thermodynamically significant conformational change in the enzyme. The dissociation constant for the binding of Mg^{2+} and P_i derived from calorimetric measurements is in good agreement with the value obtained from the kinetic studies. These results indicate that ion binding induces a conformational change in the enzyme which is a prerequisite for phosphorylation by P_i.

Membranous ATPases[1] catalyze energy transformations during ATP-driven ion transport and during ion-flux-driven ATP generation (cf. Racker, 1976). (Na^+,K^+)ATPase of the plasma membrane and (Ca^{2+})ATPase of the sarcoplasmic reticulum membrane are of particular interest because phosphorylated intermediates are formed under appropriate conditions with either ATP or P_i as substrates (Albers et al., 1963, 1968; Yamamoto and Tonomura, 1967; Lindenmayer et al., 1968; Masuda and de Meis, 1973). The carboxyl group of an aspartate residue of the protein has been identified as the recipient of the phosphate (Degani and Boyer, 1973; Post and Orcutt, 1973). Moreover, the phosphoenzyme formed with P_i was shown to transfer stoichiometrically the phosphoryl group to ADP to form ATP (Taniguchi and Post, 1975; Knowles and Racker, 1975). The net formation of ATP from P_i and ADP is catalyzed by a highly purified (Ca^{2+})ATPase under conditions that appear to rule out the formation of an ion gradient across a membrane (Knowles and Racker, 1975). The ATP formed under these conditions does not remain bound to the protein but is released into solution. These observations raised questions relating to the thermodynamics of the reactions involved.

We have proposed (Knowles and Racker, 1975) that the energy required for the condensation of P_i and ADP is derived from the binding of specific ions to the protein. To test this hypothesis, parallel kinetic and calorimetric studies with (Na^+,K^+)- and (Ca^{2+})ATPases have been carried out. In this paper we report data obtained with the (Na^+,K^+)ATPase system. We show that ligand binding to the (Na^+,K^+)ATPase monitored by calorimetric measurements can yield meaningful and accurate thermodynamic values for the binding reactions. The magnitude of the enthalpy changes for the binding of the

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Abbreviations used: Mes, 2-(*N*-morpholino)ethanesulfonic acid; NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; Tris, tris(hydroxymethyl)aminomethane.

ionic ligands, Mg^{2+} and P_i, suggests the occurrence of significant conformational changes upon binding, a conclusion supported by observations on the interaction of $NaBH_4$ with the enzyme in the presence and absence of Mg^{2+} and changes in the response of the enzyme to inhibitors in the presence of Mg^{2+} recorded here and observed previously by other investigators.

Experimental Procedure

Preparation of Enzyme. (Na^+,K^+)ATPase was purified from electric eel by a method described in the accompanying paper (Kuriki and Racker, 1976). The purified enzyme (ca. 10 mg of protein/ml in 20 mM imidazole hydrochloride, pH 7.5, and 0.25 M sucrose) was stored at −70 °C. The phospholipid content of the preparation was 1.39 μmol of phospholipid per mg of protein.

Kinetic Measurements of Phosphoenzyme Formation. The reaction mixture, containing (in 0.5 ml) 50 mM Tris-Mes[1] (pH 6.0 or pH 6.5), 5 mM $MgCl_2$, 1 mM [$^{32}P_i$]Tris-P_i (pH 6.5), and 450 μg of the enzyme protein, was incubated at 0 or 24 °C. The reaction was stopped by addition of 0.5 ml of cold 5 N trichloroacetic acid containing 20 mM phosphoric acid. The denatured protein was sedimented at 8000*g* for 10 min in the cold. The precipitate was suspended in 2 ml of cold 0.5 N trichloroacetic acid–20 mM phosphoric acid and then filtered through a type E fiberglass filter (Gelman Instrument Company, Ann Arbor, Mich.). After washing the filter with 10 ml of cold 0.5 N trichloroacetic acid–20 mM phosphoric acid and drying under an infrared lamp, the radioactivity was measured with a Nuclear Chicago gas flow counter.

Calorimetric Measurements. Heat changes associated with the binding of the ionic ligands to the ATPase were measured with a LKB Batch microcalorimeter at 24.5 °C. Prior to loading, an aliquot containing 9 mg of enzyme was diluted into 8 ml of 40 mM imidazole hydrochloride buffer (pH 6.2) and the suspension centrifuged at 156 000*g* for 30 min in a Spinco 50 Ti rotor. The pellet was suspended in 40 mM imidazole hydrochloride (pH 6.2) and the volume adjusted with the buffer to 3 ml. The enzyme solution (2 ml) was placed into the smaller section of the sample cell and 4 ml of 40 mM imidazole hydrochloride containing the ligand was placed in the other section of the sample cell of the calorimeter. Similarly, 2 ml of 40 mM imidazole hydrochloride (pH 6.2) and 4 ml of the buffer containing ligand were loaded in the smaller and larger section of the reference cell, respectively. All solutions to be mixed were adjusted to exactly the same pH before charging the calorimeter. After equilibration (1.5 to ~2 h), the two solutions were mixed by rotating the calorimetric unit. The voltage changes were recorded and the heat change was calculated by measuring the area under the voltage–time curve. The calorimeter was calibrated as described by Bolen et al. (1971). Separate experiments were performed under identical conditions to determine the heat of dilution of the enzyme.

A typical voltage–time curve for the mixing of Mg^{2+} and (Na^+,K^+)ATPase is shown in Figure 1. The time response is that expected for an instantaneous reaction. The integral heat effect was calculated from the area of the voltage–time curve using the baseline obtained by extrapolating the late time portion of the curve back to zero time.

In order to establish that the observed heat effects were not caused by nonspecific ion binding to either the enzyme or phospholipid, the following control experiments were performed. Mg^{2+} was mixed with crude phospholipid mixture obtained from microsomes of the electric organ of the electric eel. In another experiment, Mg^{2+} was mixed with a solution

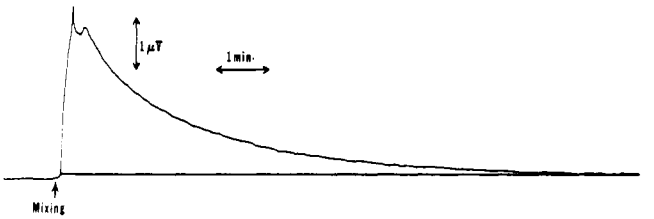


FIGURE 1: The voltage–time curve for mixing of $MgCl_2$ with (Na^+,K^+)-ATPase. The sample cell contained 2 ml of enzyme solution (5 mg/ml) and 4 ml of 15 mM $MgCl_2$ in 40 mM imidazole hydrochloride buffer. The reference cell contained 2 ml of buffer and 4 ml of 15 mM $MgCl_2$. No Na^+ or K^+ was present. After equilibration and mixing, this voltage–time curve was obtained. The observed spikes are the result of reproducible mechanical effects on the thermal elements and do not represent specific chemical processes occurring within the calorimeter cell. The area under the curve, corresponding to a total heat effect of 1.28 mcal, was calculated using the baseline shown in the figure.

of (Na^+,K^+)ATPase inactivated by storage at 25 °C for 48 h. In both cases a slow heat release was observed which appeared to be the result of ion-induced aggregation of the inactivated enzyme or lipid. The heat associated with any fast reaction occurring in these control experiments was less 0.3 mcal, which is small compared with the heat of mixing of the ligand with active enzyme. It was therefore concluded that the observed heat changes, such as those shown in Figure 1, were the result of specific ion binding to the enzyme and dilution of the components.

Incorporation of ^3H into the (Na^+,K^+)ATPase from [^3H]$NaBH_4$. The reaction mixture (0.5 ml) containing 50 mM Tris-HCl (pH 7.0), 20 mM [^{3}H]NaBH (1.1×10^7 cpm/μmol), and 0.86 mg of the enzyme was incubated at room temperature. After 30 min, 0.5 ml of 5 N trichloroacetic acid was added to stop the reaction. After centrifugation at 9200*g* for 10 min, the pellet obtained was suspended in 1.0 ml of 0.5 N trichloroacetic acid and incubated for 10 min in a boiling water bath. The precipitate was collected on a glass filter. The radioactivity retained on the glass filter was measured after successive washings of the filter with 10 ml of 0.5 N trichloroacetic acid, 2 ml of 60% ethanol, and 2 ml of 95% ethanol.

Assay of ATPase Activity. The reaction mixture (1.0 ml) containing 50 mM imidazole hydrochloride (pH 7.5), 5 mM $MgCl_2$, 60 mM NaCl, 20 mM KCl, 4 mM [γ-^{32}P]ATP and the enzyme was incubated at 37 °C. After 5 min the reaction was stopped with 50 μl of 5 N trichloroacetic acid. The preparation of [γ-^{32}P]ATP and measurements of P_i release were as described (Nelson et al., 1972).

Results and Discussion

Phosphorylation Experiments. The phosphorylation of the (Na^+,K^+)ATPase by P_i at 24 °C in the presence of Mg^{2+} was studied under a variety of conditions with respect to the ionic environment. As can be seen from Figure 2 equilibrium was attained in less than 2 min. K^+ increased and Na^+ decreased the magnitude of the equilibrium concentration of the phosphoenzyme. The formation of phosphoenzyme as a function of Tris-P_i concentration was measured at two different Mg^{2+} concentrations in the absence of K^+ and Na^+ (Figure 3). All experiments in which phosphoenzyme formation was measured as a function of [P_i] at constant [Mg^{2+}] or as a function of [Mg^{2+}] at constant [P_i] yielded a linear reciprocal relationship with the concentration of the variable ion as shown in Figures 4 and 5.

These experiments can be represented by a scheme in which the phosphorylation of the enzyme is preceded by formation

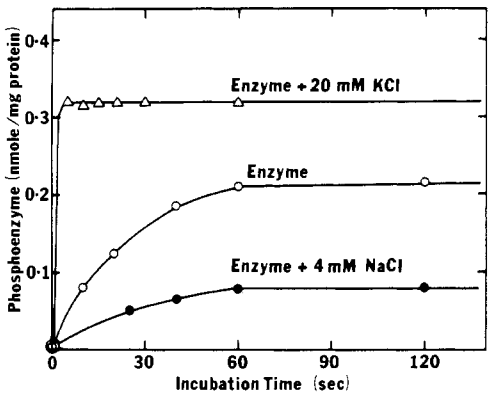


FIGURE 2: Kinetics of phosphorylation of (Na^+,K^+)ATPase by P_i with and without K^+ (or Na^+). The reaction mixture (0.5 ml) contained 50 mM Tris-Mes (pH 6.0), 5 mM $MgCl_2$, 1 mM [^{32}P]Tris-P_i (pH 6.0; 1.2×10^7 cpm/μmol), and 450 μg of the enzyme protein. After incubation with and without 20 mM KCl (or 4 mM NaCl) at 24 °C for the indicated times, phosphoenzyme was measured as described under Experimental Procedure.

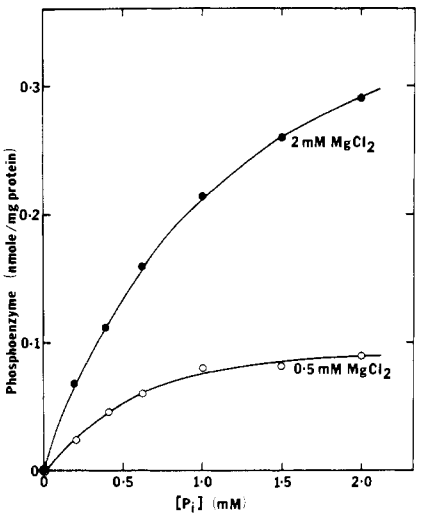
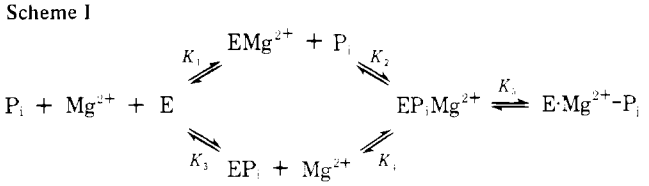


FIGURE 3: Effect of P_i concentrations on phosphorylation of (Na^+,K^+)-ATPase by P_i. The reaction mixture (0.5 ml) contained 50 mM Tris-Mes (pH 6.5), 0.5 mM or 2 mM $MgCl_2$, 450 μg of the enzyme protein, and [^{32}P]Tris-P_i (9.9×10^7 cpm/μmol). After 3 min incubation at 24 °C at the indicated concentrations, phosphoenzyme was measured as described under Experimental Procedure.

of an obligatory ternary complex of ATPase, P_i, and Mg^{2+} as shown in Scheme I. The mathematical relationship between

Scheme I



degree of phosphorylation and the various equilibrium constants and $[P_i]$ and $[Mg^{2+}]$ is given by

$$[E \cdot Mg\text{-}P] = \frac{[Mg^{2+}][P_i][E_t]K_5}{K_1K_2 + K_2[Mg^{2+}] + K_4[P_i] + (1 + K_5)[Mg^{2+}][P_i]} \quad (1)$$

where K_1, K_2, K_3, and K_4 represent the dissociation constants for Mg^{2+} or P_i at each step shown in the scheme. K_5 represents

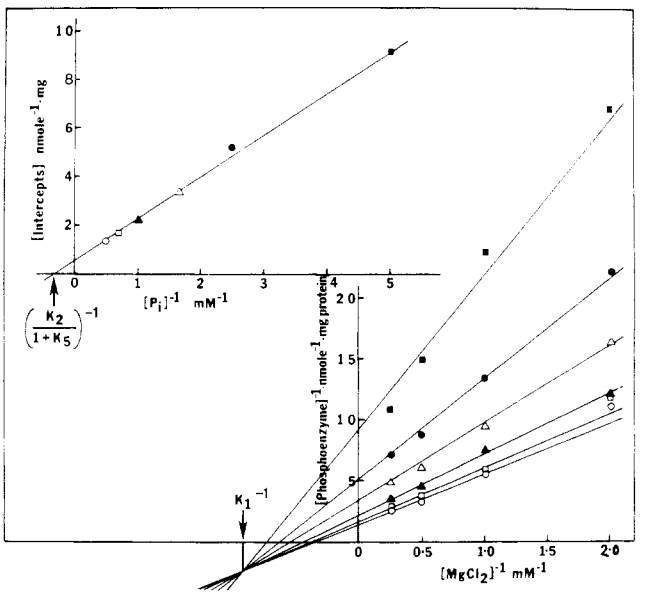


FIGURE 4: Double-reciprocal plots of phosphoenzyme formation against $MgCl_2$ concentrations. The experimental conditions were those described in the legend of Figure 3, except for the $MgCl_2$ concentrations. The inset figure shows the plots of the intercept against reciprocal of the P_i concentrations: (■) 0.2 mM P_i; (●) 0.4 mM P_i; (△) 0.6 mM P_i; (▲) 1.0 mM P_i; (□) 1.5 mM P_i; (○) 2.0 mM.

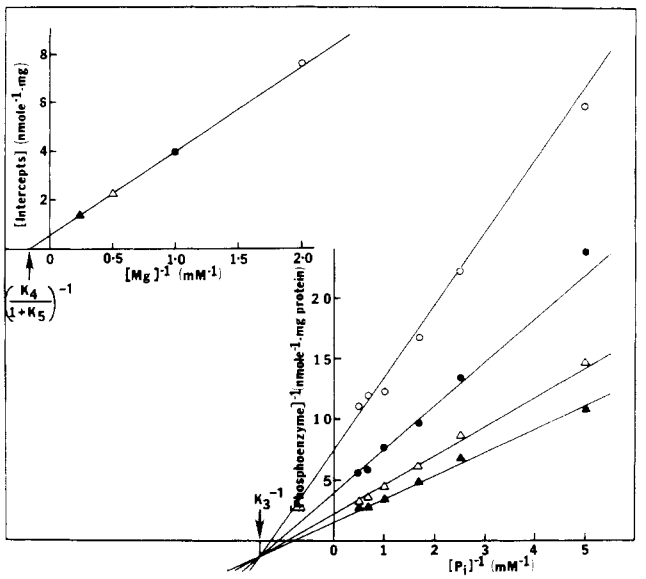


FIGURE 5: Double-reciprocal plots of phosphoenzyme formation against P_i concentrations. The experimental conditions were as described in the legend of Figure 3, except for the P_i concentrations. The inset figure shows the plots of the intercept against reciprocal of the $MgCl_2$ concentrations: (○) 0.5 mM $MgCl_2$; (●) 1.0 mM $MgCl_2$; (△) 2 mM $MgCl_2$; (▲) 4 mM $MgCl_2$.

the equilibrium constant between the phosphoenzyme and the ternary complex of Mg^{2+}, P_i, and the enzyme.

K_5 was obtained from the degree of phosphorylation extrapolated to infinite $[P_i]$ and $[Mg^{2+}]$ and assuming a molecular weight of ATPase of 140 000 (Dixon and Hokin, 1974). The other equilibrium constants were obtained by analysis of the data shown in Figures 4 and 5. The constants for the various reactions are tabulated in Table I. A comparison was made of the equilibrium binding parameters for P_i and Mg^{2+} measured calorimetrically with those obtained from the results of the phosphorylation experiment.

TABLE I: Equilibrium Constants and Apparent Thermodynamic Quantities for the Interaction of Mg^{2+} and P_i with (Na^+,K^+)ATPase.

A. Phosphorylation Results

Reaction	Dissociation Constant
$E + Mg^{2+} \rightleftarrows EMg^{2+}$	$K_1 = 1.1$ mM
$EMg^{2+} + P_i \rightleftarrows EMg^{2+}\ P_i$	$K_2 = 5.04$ mM
$E + P_i \rightleftarrows EP_i$	$K_3 = 0.67$ mM
$EP_i + Mg^{2+} \rightleftarrows EMg^{2+}\ P_i$	$K_4 = 8.67$ mM
$EMg^{2+}\ P_i \rightleftarrows EMg^{2+} - P_i$	$K_5 = 0.4$

B. Calorimetric Results[a]

Reaction	K_d (M)	$\Delta H^{\circ\prime}$ (kcal/mol)	$\Delta G^{\circ\prime}$ (kcal/mol)	$\Delta S^{\circ\prime}$ (cal mol^{-1} deg^{-1})
$E + Mg^{2+} \rightleftarrows EMg^{2+}$	0.8×10^{-3}	−42.5	−4.23	−128
$E + P_i \rightleftarrows EP_i$	3.0×10^{-3}	−49.5	−3.46	−154

[a] The thermodynamic quantities $\Delta H^{\circ\prime}$, $\Delta G^{\circ\prime}$, and $\Delta S^{\circ\prime}$ relate to the association reaction and were calculated assuming a standard state of 1 M.

General Description of the Calorimetric Experiments and Analysis of Data. The calorimetric experiments were performed by mixing an ATPase solution with ligand dissolved in the same buffer. The measured heat of mixing includes the heat of dilution of the various components plus the apparent heat of interaction of the ligand with the protein. The heat of dilution of the ligand was balanced out by an equivalent dilution experiment being carried out simultaneously in the reference cell of the calorimeter. The heat of dilution of the protein was measured in a separate experiment and subtracted from the measured heat. The resulting heat effect thus represents the apparent enthalpy change associated with ligand binding to the protein. These data were transformed into reciprocal heat units and ligand concentration and analyzed by a weighted, linear, least-squares procedure to provide estimates of the maximum heat change and the apparent binding constant of ligand for the protein system. The details of the analytical procedure have been described by Bolen et al. (1971). In all experiments, the resulting double-reciprocal plot was found to be linear, which is consistent with the existence of a single set of independent binding sites. The error associated with individual heat measurements is estimated to be ±2%. The errors in the estimated parameters, $\Delta H^{\circ\prime}$ and K, are judged to be about ±4 and ±30%, respectively.[2]

Calorimetric Studies of the Binding of Ions to the Enzyme. The apparent heat of interaction of P_i with the (Na^+,K^+)-ATPase was measured over a range of 1 to 15 mM final P_i concentration. The resulting enthalpy isotherm is shown in Figure 6A and its linear transformation to double-reciprocal coordinates in Figure 6B. The solid lines were calculated from the parameters obtained by linear, least-squares analysis of the transformed data. In this particular case, the best estimate of ΔH was −49.5 kcal/mol and $K_d = 3.0 \times 10^{-3}$ mol. It should be noted in these experiments potassium was also mixed with the free enzyme. At the concentrations used, interaction of K^+ with the enzyme produced a negligible heat effect. Mixing 30 mM KCl with the enzyme is accompanied by a $\Delta H < 5$ kcal/mol of enzyme. At higher concentration (e.g., 90 mM KCl), substantial heat release is observed, however.

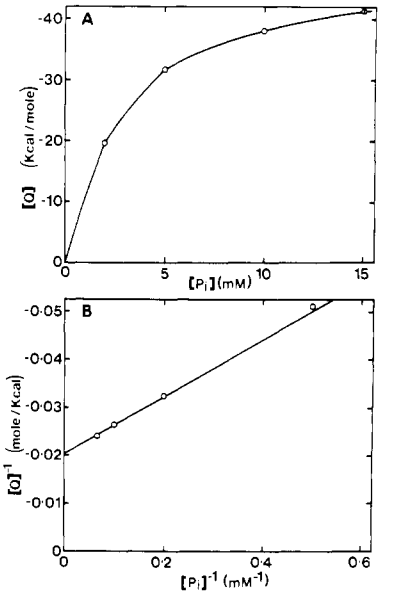


FIGURE 6: The measured enthalpy changes for the interaction of P_i with the (Na^+,K^+)ATPase. (A) Q as a function of final P_i concentration. (B) Double-reciprocal representation of the results in A. Experimental details are described in the text. The solid lines were calculated from the binding parameters given in Table I, part B.

A similar experiment was carried out in the absence of inorganic phosphate with magnesium as ligand. Analysis of the data, shown in Figure 7, yielded values of $\Delta H = -42.5$ kcal/mol and $K_d = 0.83 \times 10^{-3}$ mol.

The values of the K_d obtained from these experiments are in good agreement with similar estimates obtained from the phosphorylation data: for Mg^{2+} the estimates of K_d by the two different methods are 0.83 and 1.1 mM, respectively. For P_i they are 3.0 and 0.7 mM, respectively. This close agreement supports the model derived from the phosphorylation studies.

The magnitude of ΔH for binding of P_i and Mg^{2+} to the enzyme is extraordinarily large. These values of $\Delta H = -49.5$ and −42.5 kcal/mol can be compared with ΔH values of similar reactions involving other biological macromolecules: the ΔH for the binding of P_i to ribonuclease (Flögel et al., 1975) and for the binding of Mg^{2+} to transfer ribonucleic acid (Rialdi et al., 1972) are both approximately zero. We thus

[2] KP_i was used in the calorimetric experiments and was the only source of K^+. At the concentrations used, interaction with K^+ was negligible (less than 5 kcal/mol enzyme as judged by a control experiment using KCl). Although the K^+ effect may account for part of the observed difference in ΔH for P_i and Mg^{2+} binding, this effect is within experimental error and has been ignored. At higher K^+ concentration (60 mM), larger heat effects associated with K^+ interaction were observed.

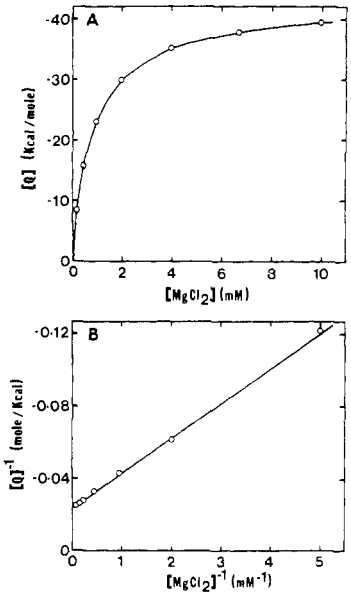


FIGURE 7: The measured enthalpy changes for the interaction of Mg^{2+} with the (Na^+,K^+)ATPase. (A) Q as a function of final Mg^{2+} concentration. (B) Double-reciprocal representation of the results in A. Experimental details are described in the text. The solid lines were calculated from the binding parameters given in Table I, part B.

TABLE II: Enthalpy Changes Associated with the Interaction of Mg^{2+} with (Na^+,K^+)ATPase in the Presence of Varying Amounts of P_i [a]

$[P_i]$ (mM)	Q_{ob} (kcal/mol)	Q_c* (kcal/mol)[b]	$Q_{ob} + Q_{P_i}$ (kcal/mol)[c]
0	−42.1	−39.1	−42.1
2	−30.2	−20.1	−49.9
5	−14.5	−9.2	−44.8
10	−6.0	−2.8	−44.6
15	−3.3	−1.3	−44.2

[a] The experiment consisted of measuring the heat of reaction of Mg^{2+} (final concentration = 10 mM) with a solution of ATPase containing varying amounts of $[P_i]$. $[P_i]$ = concentration of P_i, and Q_{ob} is the measured heat release calculated per mole of enzyme. [b] Q_c* is the calculated heat release assuming strictly competitive binding between P_i and Mg^{2+} using the binding parameters given in Table I, part B. [c] Q_{P_i} is the measured heat release upon mixing P_i with the enzyme to give a final concentration of $[P_i]$ listed in column 1. $Q_{ob} + Q_{P_i}$ is the heat for the combined experiments. Assuming synergistic effects of $P_i + Mg^{2+}$ binding $Q_{ob} + Q_{P_i} \cong$ constant as described in the text.

suspect that the ΔH associated with the binding of either P_i or Mg^{2+} to (Na^+,K^+)ATPase is the result of an induced conformational change in the enzyme system.

The apparent heat of binding Mg^{2+} to (Na^+,K^+)ATPase in the presence of various concentrations of P_i was therefore measured. The final concentration of Mg^{2+} was always 10 mM, under which condition the enzyme should always be greater than 90% saturated. The results are summarized in Table II. The concentration of P_i is recorded in the first column, and the apparent heat of Mg^{2+} binding, Q_{ob}, recorded in the second column.

The decreases in the heat changes when Mg^{2+} binding was measured in the presence of P_i can be interpreted in two ways. If there is competition between P_i and Mg^{2+}, then the apparent binding Mg^{2+} will be reduced as the P_i concentration increases. Q_c* (column III) is the apparent heat of Mg^{2+} binding calculated assuming such competition and using the binding parameters given previously. As can be seen, Q_c* is systematically less than Q_{ob} strongly suggesting such a competition is not responsible for the decrease in heat changes.

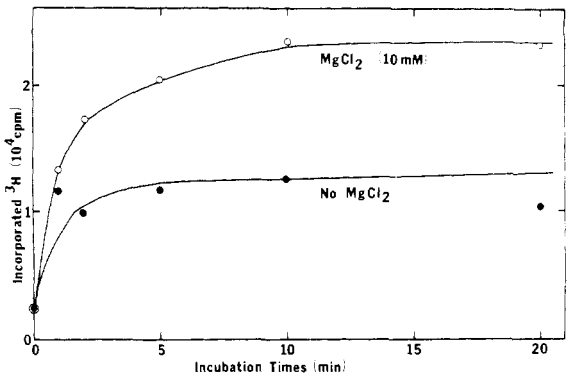


FIGURE 8: Incorporation of ^3H from [^3H]$NaBH_4$ into (Na^+,K^+)ATPase. The experimental procedures were as described under Experimental Procedure.

Another scheme to explain the results in Table II is that the enzyme exists as an equilibrium mixture of two forms and that the measured ΔH is primarily associated with the conversion of the enzyme to that form which binds P_i and Mg^{2+} best. In such a situation the heat of P_i binding at a given P_i concentration, Q_{P_i}, plus the heat associated with the addition of Mg^{2+}, Q_{ob}, should be an approximately constant value. In column IV, Table II, the sums of Q_{P_i} and Q_{ob} are tabulated. These results agree with the above hypothesis.

It is quite clear that a large enthalpy change is associated with the binding of either Mg^{2+} or P_i to the enzyme. Comparison of the magnitude of this ΔH with enthalpy changes associated with ionic interactions involving other biological macromolecules strongly suggests an induced structural alteration of the enzyme. The fact that P_i reduced the heat effect associated with Mg^{2+} binding, but did not competitively interfere with Mg^{2+} binding, is extremely strong proof that the enthalpy change is the result of an alteration of intramolecular interactions within the enzyme. The experiments described below add further support to the conclusion that such major conformational changes indeed take place.

The large heat changes associated with the interaction between Mg^{2+} and enzyme raise the interesting possibility that the acyl phosphate which is formed under these conditions may be high energy and that the driving force for its formation is actually the binding energy derived from the protein-Mg^{2+}-P_i interaction. Previous interpretations which have not taken this possibility into consideration have of necessity centered on the idea that the acyl phosphate formed from P_i is actually low energy (because of its location in a hydrophobic environment) Although this may still be correct, we can now reconsider this question in view of the new thermodynamic results. If it is true that the acyl phosphate formed in the presence of P_i is high energy; we must conclude that the interaction between Mg^{2+}, P_i, and enzyme, along with the putative conformational change of the protein, allows for the activation of the aspartate carboxyl group which serves as phosphate acceptor. The experiments described below suggest that such major conformational changes indeed take place.

Ion Dependence of the Chemical Reactivity of (Na^+,K^+)-ATPase. In the course of attempts to trap the phosphorylated intermediate of the enzyme during turnover, it was observed that Mg^{2+} has a pronounced effect on the reduction of the protein by tritium-labeled $NaBH_4$. It can be seen from Figure

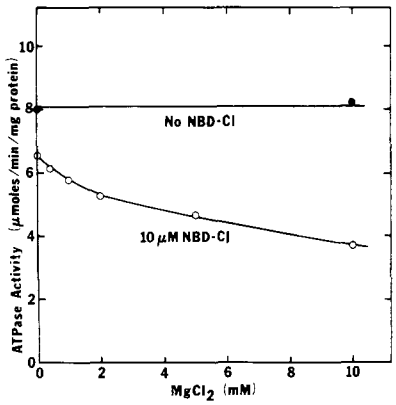


FIGURE 9: Effect of $MgCl_2$ on inhibition of (Na^+,K^+)ATPase by NBD-Cl. The reaction mixture (0.05 ml) contained 0.1 M imidazole hydrochloride (pH 7.5), 10 μM NBD-Cl, and 30 μg of the enzyme protein. After incubation for 40 h at 0 °C with and without $MgCl_2$ at the indicated concentrations, the mixture was brought to a final concentration of 0.1 M imidazole hydrochloride (pH 7.5), 5 mM $MgCl_2$, 60 mM NaCl, 20 mM KCl, 4 mM [γ-^{32}P]ATP (5.4×10^6 cpm/μmol) in 1.0 ml. The ATPase activity was measured as described under Experimental Procedure.

8 that a much larger incorporation of radioactivity was obtained in the presence than in the absence of Mg^{2+}. Similar and larger increases were observed in the presence of Mg^{2+} and K^+. Moreover, the (Ca^{2+})ATPase of sarcoplasmic reticulum responded in the same manner to the addition of Mg^{2+}. When P_i was added to the (Na^+,K^+)ATPase (in the absence of Mg^{2+}), smaller but significant increases in the reduction of the protein by borohydride were also observed. These results support the conclusion based on the calorimetric measurements that major conformational changes are induced in the protein on addition of the ionic ligands.

Another indication for an alteration in the conformation of the enzyme was obtained from studies of the inhibition of the enzyme by NBD-Cl. As shown in Figure 9 the enzyme became considerably more susceptible to inhibition when it was exposed to the inhibitor in the presence of Mg^{2+}. In neither case could the ATPase activity be recovered by addition of dithiothreitol or mercaptoethanol. In this respect the effect of the inhibitor is different from that on the ATPase from chloroplasts (Deters et al., 1975).

We have shown that the amount of Mg^{2+}-dependent phosphorylation of (Na^+,K^+)ATPase by P_i is related to the formation of a ternary complex of the enzyme, Mg^{2+}, and P_i and that the reaction is apparently associated with a ligand-induced conformational change of the enzyme. Other investigators have previously observed small but significant changes in the response of the enzyme to SH inhibitors (Hart and Titus, 1973) or to anilinonaphthalenesulfonate (Nagai et al., 1970) when Mg^{2+} was added to the enzyme. This structural change is accompanied by an enthalpy change of 40–50 kcal/mol and is such that the enzyme becomes more susceptible to reduction by $NaBH_4$ and inhibition by NBD-Cl. In a future publication we will report the results of a similar study on (Ca^{2+})ATPase from sarcoplasmic reticulum and show that it behaves in a similar fashion.

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