Phosphorylation of Calcium Adenosinetriphosphatase by Inorganic Phosphate: van't Hoff Analysis of Enthalpy Changes†

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ABSTRACT: The magnesium-dependent phosphorylation of sarcoplasmic reticulum (Ca^{2+})-ATPase by inorganic phosphate ($Mg^{2+} + P_i + E \rightleftharpoons Mg{\cdot}E{-}P$) was studied in purified leaky ATPase vesicles as a function of temperature (20–30 °C). A bireactant scheme was used to determine equilibrium constants, and the corresponding enthalpy changes ($\Delta H°_{vh}$) were determined by van't Hoff analysis. At all temperatures, the binding of P_i and Mg^{2+} to the enzyme was synergistic. The equilibrium constants showed only a modest temperature dependence, with $\Delta H°_{vh}$ varying from 3 to 13 kcal/mol. In particular, the $\Delta H°_{vh}$ for Mg^{2+} binding to the unoccupied enzyme was 3 ± 2 kcal/mol. These data contrast with a recent calorimetric study under comparable conditions (Epstein, M., Kuriki, Y., Biltonen, R., & Racker, E. (1980) *Biochemistry 17*, 5564) which reported no significant binding synergism and a $\Delta H°_{cal}$ for Mg^{2+} binding of −76 kcal/mol. A possible reason for the discrepancy between calorimetric and van't Hoff enthalpy determinations is given. In agreement with other previous work, the overall reaction was found to be accompanied by a positive entropy change.

Ca^{2+}-dependent ATPase activity in the sarcoplasmic reticulum involves a cyclic reaction scheme which is coupled to calcium transport (Tada et al., 1978; de Meis & Vianna, 1979). During catalyzed ATP[1] hydrolysis, the terminal phosphate is transferred to an aspartate residue of the enzyme, forming an acid-stable phosphate bond (Yamamoto & Tonomura, 1968; Makinose, 1969; Inesi et al., 1970). Subsequently, the enzyme dephosphorylates, releasing inorganic phosphate (P_i). This reaction cycle is completely reversible (Makinose & Hasselbach, 1971). The ATPase may be phosphorylated by the addition of free inorganic phosphate in the presence of magnesium (Masuda & de Meis, 1973), and ATP can then be generated from ADP (de Meis & Carvalho, 1974; Knowles & Racker, 1975). In the absence of Ca^{2+}, addition of P_i and Mg^{2+} results in a true equilibrium between free enzyme (E) and phosphorylated enzyme (E–P).

Kolassa et al. (1979) demonstrated that the formation of the ternary complex Mg·E–P occurs by random addition of substrates according to the scheme of Figure 1 and equilibrium constants for each step in the scheme were determined by Punzengruber et al. (1978). The identical scheme was used by Epstein et al. (1980) and had been previously assumed by Kuriki et al. (1976) for the formation of a similar phosphoenzyme complex by (Na^+,K^+)-ATPase. We have used the same scheme for analysis of the data reported in this paper.

Generating phosphoenzyme (E–P) from P_i is an unusual reaction since acyl phosphate bond formation generally requires a high energy phosphate source. Racker and co-workers (Kuriki et al., 1976; Racker, 1977; Epstein et al., 1977, 1980) have proposed that the binding of ions (particularly Mg^{2+} and P_i) to the protein induces significant conformational changes which stabilize E–P formation. This proposal was based in part on calorimetric studies (of both (Na^+,K^+)-ATPase and (Ca^+)-ATPase) which indicated that the binding of either Mg^{2+} or P_i to the enzyme is accompanied by an extraordinarily large negative enthalpy change. In a recent calorimetric study Epstein et al. (1980) reported that in terms of Figure 1, ΔH_1 and ΔH_2 had values of −23 and −76 kcal/mol, respectively.

An enthalpy change of −76 kcal/mol, by the van't Hoff relation

$$\frac{d \ln K}{d(1/T)} = \frac{-\Delta H°_{vh}}{R} \quad (1)$$

where $\Delta H°_{vh}$ is the van't Hoff enthalpy at standard state, would correspond to a large temperature dependence of the equilibrium constant for the reaction: at 20 °C, K should decrease by a factor of ~74 for a ΔT of +10 °C. This would represent an unusually high temperature dependence for an ion–protein interaction. We have therefore measured the equilibria in the phosphorylation reaction as a function of temperature under conditions similar to those used in the calorimetric studies (e.g., absence of K^+), and the results are reported in this paper. We find that K_1 and K_2 increase with temperature instead of decreasing. The associated enthalpy changes, determined by the van't Hoff relation, are positive and small in magnitude. Enthalpy changes for other reactions in the scheme of Figure 1 were also found to be relatively small. The overall effect of increasing temperature is to raise the level of phosphoenzyme, in agreement with what was observed by Kanazawa (1975) in studies of the overall reaction for a solubilized ATPase.

Materials and Methods

Preparation of (Ca^{2+})-ATPase Vesicles. Sarcoplasmic reticulum vesicles were prepared from homogenized rabbit white skeletal muscle and partially extracted with recrystallized deoxycholate according to Meissner et al. (1973). Gel electrophoresis indicated that ~95% of the protein in ATPase vesicle was (Ca^{2+})-ATPase (le Maire et al., 1976). Protein concentrations were determined by the method of Lowry et al. (1951) and by A_{280} with an extinction coefficient of $E^{1cm}_{0.1\%} = 1.2$ (Hardwicke & Green, 1974). ATPase activity of the vesicle preparations was determined at 37 °C using a coupled enzyme assay (Warren et al., 1974) as previously described (Dean & Tanford, 1977). Activities of acceptable vesicle

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[1] Abbreviations used: ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; P_i, inorganic phosphate (ortho); Cl_3CCO_2H, trichloroacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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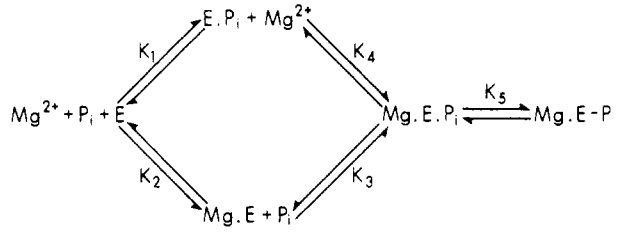

FIGURE 1: Bireactant scheme for the Mg^{2+} and P_i dependency of phosphoenzyme formation. All equilibrium constants are leading in the direction of Mg·E–P formation.

preparations were 16–19 μmol of ATP hydrolyzed min^{-1} (mg of protein)$^{-1}$.

Measurement of Phosphoenzyme Formation. The pH of all solutions was adjusted to 6.2. $^{32}P_i$ solutions were centrifuged (Beckman Airfuge, 30 psi, ~160000*g*, ≥20 min, cellulose nitrate tubes, 22 °C). The top two-thirds of the solution was removed and used in phosphorylation assays. This procedure removed precipitable radioactive contaminants which contribute only ~1% to the radioactivity of stock solutions but contribute significantly to nonspecifically associated counts on precipitated vesicles.

The reaction mixture was 34 mM Mes–Tris (pH 6.2) and 5 mM EGTA and contained ATPase vesicles, $MgCl_2$, and $^{32}P_i$ [50 Ci/mol, isotropically diluted with H_3PO_4 (ortho)] at concentrations indicated in the figure legends. The total reaction volume was 75 μL. The reaction mixture (55 μL) without P_i was equilibrated (≥10 min) in polypropylene microcentrifuge tubes (500 μL) situated in a water-perfused aluminum block. The block was maintained at a constant temperature (±0.05 °C) using a proportional temperature control regulating a heating bath cooled at a constant rate with a separate cooling bath. The reaction was initiated by addition of $^{32}P_i$ (20 μL, temperature equilibrated) and allowed to reach equilibrium for 10 s. (Phosphoenzyme formation reached a constant level within 5 s of reaction and maintained a constant value for at least 20 s.) The reaction was quenched by the rapid addition of 75 μL of ice-cold 10% (w/v) Cl_3CCO_2H, and 200 mM phosphoric acid (ortho). The precipitated protein was centrifuged 2.5 min at 12800*g* in an Eppendorf 3200 microcentrifuge. The supernatant was aspirated, and the pellet was washed with 150 μL of quenching solution and centrifuged 1 min at 12800*g*. The supernatant was aspirated and the wash repeated three additional times. The tip of the tube containing the resultant protein pellet was cut off with a razor and placed in a scintillation vial to which 1 mL of H_2O and 10 mL of Aqueous Counting Scintillant (Amersham) were subsequently added. The thoroughly vortexed scintillation vial was counted in a Beckman LS-100-C liquid scintillation counter.

"Nonspecific" $^{32}P_i$ incorporation was assayed in parallel at identical P_i concentrations by incubating the vesicles in the absence of $MgCl_2$ and in the presence of 15 mM EGTA. This method gave the same results as that obtained when phosphorylation in the presence of $MgCl_2$ was attempted with heat-denatured vesicles or Cl_3CCO_2H-denatured vesicles. Control studies indicated that ~90% of the applied protein was recovered after Cl_3CCO_2H precipitation and subsequent washes.

The phosphate concentrations of stock solutions were determined against a standard curve by use of the phosphate assay of Bartlett (1959). The Mg^{2+} concentrations of stock $MgCl_2$ solutions were determined against standard curves by use of atomic absorption (Varian Techtron AA-5 spectrophotometer, Carbon Rod Atomizer 63). The concentration of Mg^{2+} remaining associated with the vesicles prior to incubation in the reaction mixture was 4×10^{-5} mol/g protein. The free concentrations of P_i and Mg^{2+} at pH 6.2 were determined, using a Newton-Raphson algorithm, from the total applied concentrations of H_3PO_4, EGTA, and $MgCl_2$ and the appropriate association constants (Vianna, 1975; Punzengruber et al., 1978). In the temperature dependency studies, free phosphate and magnesium were 94–98% of the total applied ion concentrations (i.e., correction for complexed Mg and P_i did not significantly affect the results).

The observed levels of E–P formation were corrected for the dissociation of E–P in Cl_3CCO_2H. This correction was made by extrapolating back to the initial point of quenching with a decay rate of 0.0031 min^{-1} (see Results) and the total Cl_3CCO_2H exposure time. All results are expressed in terms of the molar ratio $[E–P]/[E_t]$, where $[E_t]$ is the molar concentration of ATPase polypeptide chains. The measured protein concentration (in mg/mL) was corrected for the presence of non-ATPase protein (5%) and converted to molar units assuming a polypeptide molecular weight of 119 000 (Rizzolo et al., 1976). The resultant data were fitted by regression analysis to linear functions described below.

Results

The stability in Cl_3CCO_2H of the covalently bonded phosphate was assayed during extended periods of washing with Cl_3CCO_2H. After the initial removal of trapped, nonbonded $^{32}P_i$ (three washes), the decay of specifically incorporated $^{32}P_i$ was followed. Control studies indicated that the decay of phosphoenzyme was not due to a loss of protein. The rate of phosphoenzyme decay could be fitted to a first-order model consistent with an irreversible loss of bound phosphates (data not shown). Analysis of the decay of phosphoenzyme formed from varied initial free P_i concentrations (0.01–4 mM P_i) yielded a mean decay rate of 0.0031 min^{-1} ($t_{1/2}$ ~ 3.75 h). The decay rate was not significantly dependent on the level ($[E–P]/[E_t]$) of phosphoenzyme formed or the initial free P_i concentration. Observed levels of $^{32}P_i$ incorporation were corrected for this loss during washing (see Materials and Methods), the amount of correction being about 10%.

At a constant concentration of magnesium, E–P formation increases with applied free P_i in a saturable manner (Figure 2). Since less than 0.2% of the applied P_i was incorporated into the protein, at all $[P_i]$ studied, E–P formation was plotted as a function of total free phosphate (adjusted for loss due to complexation as $MgHPO_4$; see Materials and Methods). Nonspecific P_i incorporation into the protein was found to be proportional to the applied P_i concentration and comprised only a very small fraction of the total phosphorylated precipitate (Figure 2).

At a constant concentration of P_i, E–P formation at first increases with Mg^{2+} concentration, as expected from the reaction scheme, but the E–P level decreases at very high Mg^{2+} concentrations, as shown in Figure 3. A similar observation was made by Kanazawa (1975) in a study of a solubilized (Ca^{2+})-ATPase preparation. A small portion of the decrease in $[E–P]/[E_t]$ at high $[Mg^{2+}]$ is due to reduced free $[P_i]$ as a result of $MgHPO_4$ formation. However, the reaction scheme of Figure 1 must be modified to fully account for the major portion of this effect, and the type of modification required will be considered under Discussion. The subsequent results will show that data at $[Mg^{2+}] \leq 12$ mM obey linear equations based on the scheme of Figure 1, and the bulk of our results were obtained at concentrations below this limit. Previous studies demonstrating the applicability of the scheme (Figure 1) were similarly confined to relatively low $[Mg^{2+}]$ concentrations; the maximal concentration employed by Kolassa et

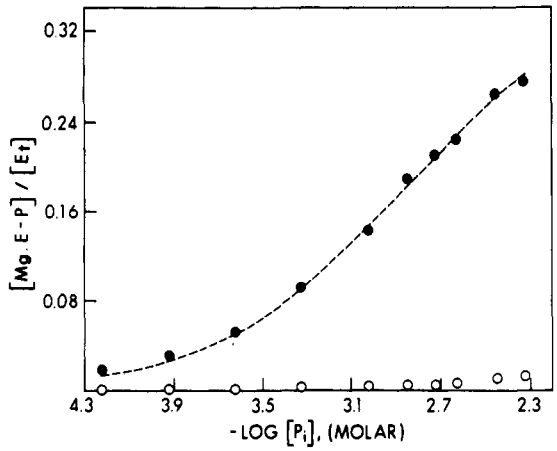

FIGURE 2: Phosphoenzyme formation as a function of varied $[P_i]$ at constant $[Mg^{2+}]$. Mg·E–P was generated and specific (●) and nonspecific (○) phosphate incorporation determined as described under Materials and Methods using ATPase vesicles (1 mg/mL), $MgCl_2$ (20 mM), and $^{32}P_i$ (0.067–5.33 mM, 70 Ci/mol). The above data are plotted as $[Mg{\cdot}E{-}P]/[E_t]$ vs. $-\log [P_i]$ where $[P_i]$ is the molar concentration of free P_i. The curve is a theoretical curve based on the values of K_{app} (699 M^{-1}) and n_{app} (0.364) determined from a Scatchard plot as described under Results. Each point represents the mean of triplicate determinations with a maximum standard deviation of <7%.

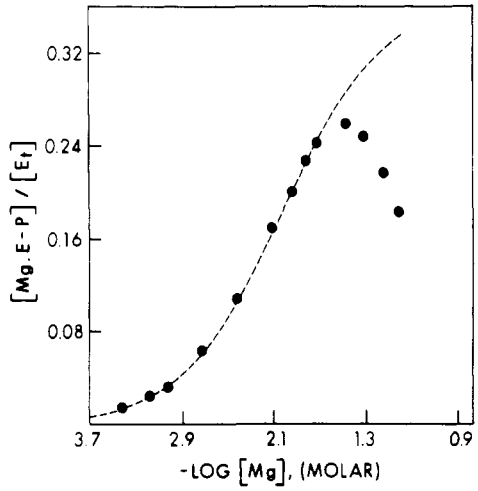

FIGURE 3: Phosphoenzyme formation as a function of varied $[Mg^{2+}]$ at constant $[P_i]$. Mg·E–P was generated as described under Materials and Methods using ATPase vesicles (1 mg/mL), $^{32}P_i$ (2 mM, 50 Ci/mol), and $MgCl_2$ (0.4–100 mM). The data are plotted as $[Mg{\cdot}E{-}P]/[E_t]$ vs. $-\log [Mg]$ where [Mg] is the molar concentration of free magnesium. The curve is a theoretical curve obtained as described under Results using K_{app} and n_{app} determined from a Scatchard plot of the data. Each point represents the mean of triplicate determinatons, with a maximum standard deviation of <5%.

al. (1979) and Epstein et al. (1980) was 20 and 25 mM, respectively, and for the Na^+,K^+ pump studied by Kuriki et al. (1976) it was 4 mM.

The reaction scheme of Figure 1 leads to

$$\frac{[Mg{\cdot}E{-}P]}{[E_t]} = \frac{[Mg][P_i]K_5 n}{(K_2K_3)^{-1} + ([Mg]/K_3) + ([P_i]/K_4) + (1 + K_5)[Mg][P_i]} \quad (2)$$

for the extent of phosphoenzyme formation as a function of free $[Mg^{2+}]$ and $[P_i]$, where K_1–K_4 are association constants, K_5 is the equilibrium constant for formation of Mg·E–P, and n is the number of phosphorylation sites per polypeptide chain. The equilibrium constants in eq 2 can be determined graphically from data obtained by varying one substrate and keeping the other constant. At *constant* Mg^{2+} and variable P_i, eq 2 reduces to a bimolecular equilibrium equation

$$\frac{[Mg{\cdot}E{-}P]}{[E_t]} = \frac{n_{app}K_{app}C}{1 + K_{app}C} \quad (3)$$

where C represents $[P_i]$ and

$$n_{app} = \frac{nK_5[Mg]}{K_4^{-1} + (1 + K_5)[Mg]} \quad (4)$$

$$K_{app} = \frac{K_4^{-1} + (1 + K_5)[Mg]}{(K_2K_3)^{-1} + K_3^{-1}[Mg]} \quad (5)$$

Similarly, at *constant* P_i and variable Mg^{2+}, eq 3 applies, with C representing $[Mg^{2+}]$ and

$$n_{app} = \frac{nK_5[P_i]}{K_3^{-1} + (1 + K_5)[P_i]} \quad (6)$$

$$K_{app} = \frac{K_3^{-1} + (1 + K_5)[P_i]}{(K_1K_4)^{-1} + K_4^{-1}[P_i]} \quad (7)$$

Equation 3 predicts linearity of double-reciprocol plots and Scatchard plots, and observation of linearity is a test for the validity of eq 2 as well as a means for calculating the constants n_{app} and K_{app}.

It should be noted that full saturation of the phosphorylation sites cannot occur in a system obeying the mechanism of Figure 1 unless K_5 is infinite. The maximal degree of phosphorylation ($[Mg{\cdot}E{-}P]$ extrapolated to $[Mg] = [P_i] = \infty$) therefore yields $[Mg{\cdot}E{-}P]_{max}/[E_t] = nK_5/(1 + K_5)$. However, n cannot be evaluated from the experimental data. We have assumed one site per polypeptide chain, and under Discussion will show that no other choice is consistent with the results obtained at all three temperatures. One site per chain corresponds to $n = 1.0$ if the parameters used to calculate $[E_t]$ are correct. Possible deviations between true polypeptide molecular weight and the assumed value of 119 000 and the uncertainities in the amount of non-ATPase protein lead to an uncertainty of about ±0.1 in the value of n that corresponds to one site per chain.

Replotting the data of Figure 2 according to Scatchard (1949) revealed a nonlinear portion, resulting from elevated E–P formation at low ion concentrations (data not shown). This deviation from linearity was obtained consistently, and a similar deviation from linearity at low ion concentrations may be observed in the double-reciprocal plots of others (Epstein et al., 1980; Punzengruber et al., 1978; Rauch et al., 1977; Kuriki et al., 1976). The linear portion of the Scatchard plot was used to estimate K_{app} and n_{app} and hence a theoretical curve for the data of Figure 2. The theoretical curve shows that the deviation from linearity at low $[P_i]$ represents an insignificant fraction of the total incorporated ^{32}P. Similar results were obtained at other constant $[Mg^{2+}]$ concentrations, and the "extra" phosphoprotein will hereafter be ignored.

No deviations from linearity were observed in double-reciprocal or Scatchard plots of the data of Figure 3 (or similar data at other temperatures and P_i concentrations >0.5 mM) provided that only results at $[Mg^{2+}] \leq 12$ mM were used, as is exemplified by Figures 4A and 5A.

The parameters n_{app} and K_{app} of eq 3–7 are conveniently obtained by use of double-reciprocal plots or Scatchard plots. The double reciprocal plots are shown in Figures 4A and 5A, and are linear as required by the reaction model. Scatchard plots (not shown) were also used on all data sets and gave results consistent with those obtained from double-reciprocal

Table I: Temperature Dependence of Equilibrium Constants[a]

temp (°C)	K_1 (M^{-1})	K_2 (M^{-1})	K_3 (M^{-1})	K_4 (M^{-1})	K_5	$\alpha(P_i)$ (K_3/K_1)	$\alpha(Mg)$ (K_4/K_2)	max [Mg-P]/[E_t][c]
20	72 ± 10	114 ± 7	532 ± 62	833 ± 160	0.79 ± 0.05	7.4	7.3	0.44
25	105 ± 23	128 ± 17	524 ± 74	637 ± 130	0.93 ± 0.06	5.0	5.0	0.48
30	141 ± 16	132 ± 16	426 ± 85	397 ± 77	1.21 ± 0.15	3.0	3.0	0.55
20[b]	139 ± 36	114 ± 13	654 ± 85	529 ± 70	0.60 ± 0.08	4.7	4.6	0.37

[a] Average values ± standard deviation of mean obtained at each temperature from four different linearized plots as described under Results. [b] From Punzengruber et al. (1978); pH 7, assumed peptide chain molecular weight = 100 000; average ± standard deviations of three reported data sets. [c] $[P_i] \rightarrow \infty$, $[Mg] \rightarrow \infty$.

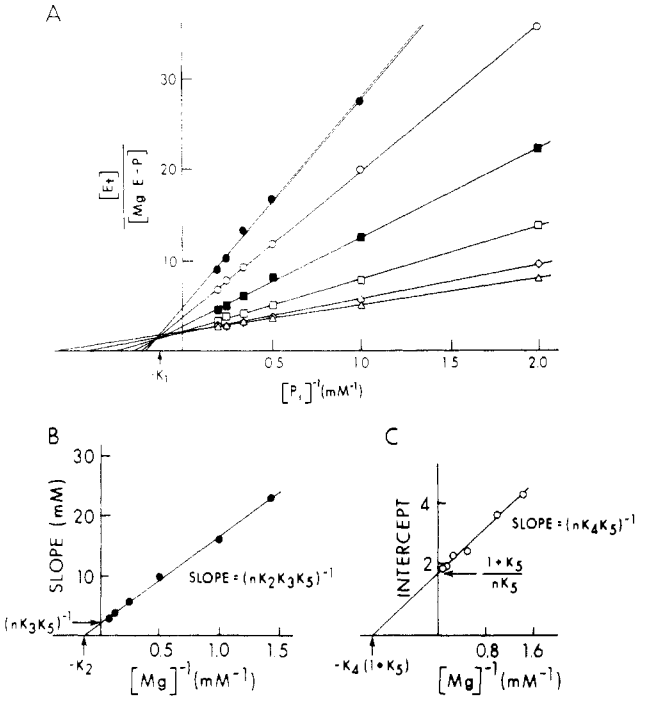

FIGURE 4: (A) Double-reciprocal plots of phosphoenzyme formation at constant [Mg] and varied [P_i]. Data obtained at 25 °C as previously described with $MgCl_2$ concentrations of (●) 0.7 mM, (○) 1 mM, (■) 2 mM, (□) 4 mM, (◊) 8 mM, and (△) 12 mM. Each point represents the mean of triplicate determinations, with a maximum standard deviation of <5%. (B) Slopes of the lines in (A) are plotted against the reciprocal magnesium concentration. (C) y intercepts of the lines in (A) are plotted against the reciprocal magnesium concentration.

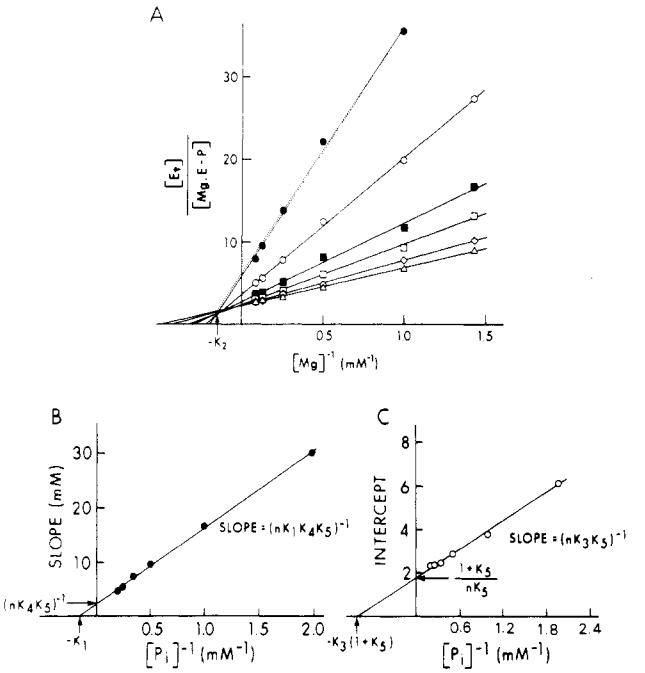

FIGURE 5: (A) Double-reciprocal plots of phosphoenzyme formation at constant [P_i] and varied [Mg]. Data obtained at 25 °C as previously described with P_i concentrations of (●) 0.49 mM, (○) 0.99 mM, (■) 1.97 mM, (□) 2.91 mM, (◊) 3.91 mM, and (△) 5 mM. Each point represents the mean of triplicate determinations with a maximum standard deviation of <5%. (B) Slopes of the lines in (A) are plotted against the reciprocal P_i concentration. (C) y intercepts of the lines in (A) are plotted against the reciprocal P_i concentration.

plots. All equilibrium constants of the scheme of Figure 1 can be obtained from secondary plots of the slopes and intercepts of Figures 4A and 5A vs. $1/[Mg^{2+}]$ or $1/[P_i]$ as shown by Figures 4B,C and 5B,C. The procedure used in the double-reciprocal plots is identical with that employed by Punzengruber et al. (1978) and Kuriki et al. (1976). Again, the same information can be obtained from the slopes and intercepts of Scatchard plots, and the summary of all data in Table I represents averages of the values of both procedures.

The points of common intersection of the plots in Figures 4A and 5A fall above the reciprocal substrate axis, indicating that the binding of one substrate increases the affinity of the enzyme for the second substrate (Segel, 1975). This affinity change may be quantitated by the ratio of the equilibrium constants where

$$\alpha(P_i) = K_3/K_1 \quad (8)$$

and

$$\alpha(Mg) = K_4/K_2 \quad (9)$$

The scheme in Figure 1 requires that

$$K_1K_4 = K_2K_3 \quad (10)$$

therefore

$$\alpha(P_i) = \alpha(Mg) = \alpha \quad (11)$$

In terms of association constants, α is greater than 1 when the affinity increases upon ligand binding.

Experiments identical with those of Figures 4 and 5 (25 °C) were conducted at 20 and 30 °C and the data analyzed as described above. The equilibrium constants obtained at the three temperatures are given in Table I. Also listed are values reported by Punzengruber et al. (1978) obtained at 20 °C, pH 7. At all temperatures α was greater than 1, and within each temperature $\alpha(P_i) = \alpha(Mg)$, in agreement with the reaction scheme of Figure 1. Differences between our data and those of Punzengruber et al. may be related to the pH dependency of E–P formation (Masuda & deMeis, 1973; Beil et al., 1977). Contrary to what would have been predicted from calorimetry data (Epstein et al., 1977, 1980; Racker, 1977; Kuriki et al., 1976), there was only a modest change in equilibrium constants with temperature. Additionally, the synergism between Mg^{2+} and P_i binding, as characterized by α, decreased with increasing temperature. The equilibrium constants for the reaction steps were plotted according to the van't Hoff relation (eq 1). The van't Hoff plots for the equilibrium constants are shown in Figure 6. Table II gives the $\Delta H°_{vh}$ values obtained for all of the reaction steps of Figure 1. For comparison, the $\Delta H°_{cal}$ values reported from calorimetric studies of Mg^{2+} and P_i binding to (Ca^{2+})-ATPase are also given. In contrast to the calorimetric data, van't Hoff analysis indicates that no step

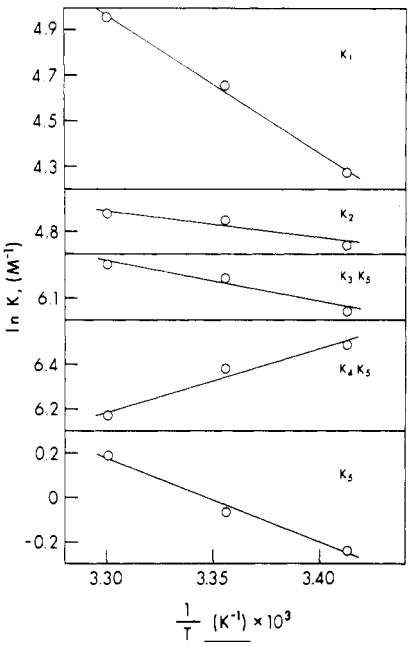

FIGURE 6: van't Hoff plots of equilibrium constants given in Table I. The products K_3K_5 and K_4K_5 were used for this plot because they are obtained directly from Figures 4 and 5 and subject to less experimental error than K_3 and K_4 (see Table I). The slopes yield $\Delta H_3 + \Delta H_5$ and $\Delta H_4 + \Delta H_5$, respectively, and ΔH_3 and ΔH_4 are obtained by subtracting ΔH_5.

Table II: van't Hoff Analysis of Temperature Dependence of Equilibrium Constants

reaction	ΔH_{vh}[a] (kcal/mol)
$E + P_i \overset{K_1}{\rightleftharpoons} E{\cdot}P_i$	12 ± 2
$E + Mg^{2+} \overset{K_2}{\rightleftharpoons} Mg{\cdot}E$	3 ± 2
$Mg{\cdot}E + P_i \overset{K_3}{\rightleftharpoons} Mg{\cdot}E{\cdot}P_i$	−4 ± 3
$E{\cdot}P_i + Mg^{2+} \overset{K_4}{\rightleftharpoons} Mg{\cdot}E{\cdot}P_i$	−13 ± 4
$Mg{\cdot}E{\cdot}P_i \overset{K_5}{\rightleftharpoons} Mg{\cdot}E{-}P$	7 ± 2
enthalpy change determined by calorimetry[b]	**ΔH_{cal} (kcal/mol)**
$E + P_i \overset{K_1}{\rightleftharpoons} E{\cdot}P_i$	−23
$E + Mg^{2+} \overset{K_2}{\rightleftharpoons} Mg{\cdot}E$	−76
$Mg{\cdot}E + P_i \overset{K_3}{\rightleftharpoons} Mg{\cdot}E{\cdot}P_i$	−9
$E{\cdot}P_i + Mg^{2+} \overset{K_4}{\rightleftharpoons} Mg{\cdot}E{\cdot}P_i$	−35

[a] Values are the mean ± standard deviations of values obtained from four different sets of equilibrium constants representing different linearized plots. [b] Epstein et al. (1980).

in the reaction sequence is characterized by very large negative enthalpy changes. $\Delta H°_{vh}$ values for Mg^{2+} and P_i binding were small and positive. In general agreement with others (Kanazawa, 1975; Beil et al., 1977; Rauch et al., 1977), summation of the reaction heats suggests that the overall reaction ($E + Mg^{2+} + P_i \rightleftharpoons Mg{\cdot}E{-}P$) is characterized by a positive enthalpy change ($\Delta H°_{vh} \sim 6$ kcal/mol).

Discussion

The equilibrium constants obtained in this study at 20 °C are in good agreement with the earlier work of Punzengruber et al. (1978) as shown in Table I. Differences between the systems used in these two studies and others (pH, protein composition and specific activity of vesicle preparations, and assumed molecular weight of the enzyme, etc.) presumably account for the minor discrepancies that are observed.

It is known that the ATPase protein can exist in two conformations, one (E_1) which can be phosphorylated by ATP but not by P_i, the other (E_2) which undergoes the reaction described in this paper (de Meis & Vianna, 1979). The unliganded protein E is thus actually a mixture of the two forms, and the equilibrium between them

$$E_1 \overset{K_0}{\rightleftharpoons} E_2 \qquad (12)$$

should formally be made a part of the scheme of Figure 1. If this is done, the experimental equilibrium constants K_1 and K_2 would be replaced by $K_1'/[1 + (1/K_0)]$ and $K_2'/[1 + (1/K_0)]$, respectively, where K_1' and K_2' are the equilibrium constants for reaction with E_2 form alone. It is likely that E_2 is the stable state of the enzyme in the absence of Ca^{2+} (i.e., $K_0 > 1$) so that the measured K_1 and K_2 are probably not substantially different from K_1' and K_2'. This question is, however, irrelevant for a comparison between the experimental data of this paper and other work cited. Previous studies (Epstein et al., 1980; Punzengruber et al., 1978) ignored the existence of two conformational states of the unliganded protein (Figure 1), which is formally equivalent to assuming $K_0 > 1$.

Phosphorylation Sites per Polypeptide Chain. For experiments that are done at a single temperature, any value of n greater than $[Mg{\cdot}E{-}P]_{max}/[E_t]$ can be used since

$$\frac{[Mg{\cdot}E{-}P]_{max}/[E_t]}{n - [Mg{\cdot}E{-}P]_{max}/[E_t]} = K_5 \qquad (13)$$

and K_5 must be positive. This fact was verified by Punzengruber et al. (1978) who reported that n could be increased or decreased without affecting the goodness of fit of calculated parameters to their experimental data obtained at 20 °C (however, α values were affected by changes in n). Since it is unlikely that the heat capacity of the protein varies significantly between 20 and 30 °C, the linearity of the van't Hoff plots (Figure 6) may provide a criterion by which to judge the validity of the choice of n. With $n = 1$, all of the plots are slightly nonlinear but the deviation from linearity is well within the experimental uncertainties of the K values. Increasing the value of n ($n = 1.1, 1.2$) gives slightly better linearity, suggesting that the peptide chain molecular weight may be less than 119000. However, decreasing values of n give progressively greater nonlinearity, and any value approaching one site per two chains yields plots of unacceptably high curvature. Taken together, these data strongly favor one active site per peptide chain, and mechanisms suggesting that only every second transport protein can accept a phosphate residue (Beil et al., 1977) would appear to be inappropriate.

Effect of High Mg^{2+} Concentration. At high concentrations of Mg^{2+} the level of $Mg{\cdot}E{-}P$ formed at constant P_i decreases, as shown in Figure 3. The simplest explanation for this is to invoke the equilibrium between E_1 and E_2 (eq 12). The form E_1 contains the high-affinity binding site for Ca^{2+} (de Meis & Vianna, 1979), and stabilization of E_1 by the formation of Ca_2E_1 is responsible for the inhibition of phosphorylation by P_i in the presence of Ca^{2+}. However, these sites can also bind Mg^{2+} with low affinity (Kalbitzer et al., 1978; Hasselbach, 1979), and an additional site for Mg^{2+} has been reported by Dupont (1980). High concentrations of Mg^{2+} would therefore be expected to be inhibiting by virtue of the reaction $3Mg^{2+} + E_1 \rightleftharpoons Mg_3E_1$. The results of Figure 3 can be accounted for with a reasonable value for the equilibrium constant of this

reaction, but a detailed study over a wide range of P_i concentrations has not been made.

Interdependence of Mg^{2+} and P_i Binding. Our data and those of Punzengruber et al. (1978) indicate that Mg^{2+} and P_i bind to (Ca^{2+})-ATPase synergistically. This synergism may be temperature and pH dependent (Table I) (i.e., it decreases with increasing temperature or pH). At 20 °C, pH 6.2, the binding of one ion can increase the affinity of the enzyme for the other ion by greater than 7-fold ($\alpha = 7.3$). This corresponds to a free energy change in ion binding of only 1 kcal/mol.[2]

Enthalpy Changes Associated with Mg^{2+} and P_i Binding. The reaction steps of Figure 1 have only a relatively slight temperature dependence, and $\Delta H°_{vh}$ values are in the normal range expected of biochemical reactions and ion binding to macromolecules (Flogel et al., 1975; Rialdi et al., 1972). In particular, ΔH_1 and ΔH_2, which were assigned values of −23 and −76 kcal/mol, respectively, on the basis of calorimetric data, are not only much smaller in magnitude but even of opposite sign. It is impossible to account for the discrepancy between van't Hoff and calorimetric values without introducing additional steps into the reaction scheme of Figure 1. The existence of the equilibrium of eq 12 cannot of course be a factor because the temperature dependence of K_0 is an implicit part of ΔH_1 and ΔH_2 in both kinds of studies. A possible clue to the discrepancy is provided by examination of the experimental procedures involved in the two enthalpy determinations.

In contrast to the van't Hoff data, ΔH_{cal} determinations with (Na^+,K^+)-ATPase and (Ca^{2+})-ATPase required lengthy (1–2 h) preincubations of the enzyme in the absence of substrate (Kuriki et al., 1976; Epstein et al., 1980). Incubation of an enzyme in the absence of substrates may lead to a slow reversible denaturation of the enzyme (Pace & McGrath, 1980; Citri, 1973), and we have evidence for instability in the unliganded form of (Ca^{2+})-ATPase (unpublished). Transitions between active and inactive states often involve large positive enthalpy changes (Privalov & Khechinashvili, 1974). Reversal of denaturation by addition of Mg^{2+} or P_i might in that case give an anomalously large negative ΔH for the apparent enthalpy change of ion binding.

Entropy Change of Phosphorylation. The free energy change for the overall reaction at 25 °C ($E + Mg^{2+} + P_i \rightleftharpoons Mg{\cdot}E{-}P$) is $\Delta G° \sim -6.5$ kcal/mol; however, $\Delta H = +6$ kcal/mol. The overall entropy change must therefore be positive ($\Delta S° = +42.1$ eu at 25 °C), in agreement with the earlier studies of Kanazawa (1975). This does not necessarily mean that Mg·E–P is a disordered state since release of bound water molecules provides an alternative source of positive entropy change.

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[2] In analogous studies, Kuriki et al. (1976) reported that P_i and Mg^{2+} binding to (Na^+,K^+)-ATPase was antagonistic, with a greater than 7-fold decrease in enzyme affinity for P_i upon binding Mg^{2+}. The data of Epstein et al. (1977, 1980) give $\alpha \simeq 1$ for their (Ca^{2+})-ATPase system.