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Phosphorylation of Calcium Adenosinetriphosphatase by Inorganic Phosphate: Reversible Inhibition at High Magnesium Ion Concentrations†

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ABSTRACT: Magnesium stimulates phosphorylation of the calcium pump protein of the sarcoplasmic reticulum by inorganic phosphate, but the effect is reversed by high [Mg^{2+}]. This reversal is readily explained in terms of the generally accepted existence of two conformational states of the enzyme, E_1 and E_2. E_2 is the form of the enzyme that can be phosphorylated by P_i, and it has one binding site for Mg^{2+}. E_1 is the form of the enzyme that has two high-affinity Ca^{2+} binding sites, and it is phosphorylated by ATP when Ca^{2+} is bound. Mg^{2+} can bind weakly to the two Ca^{2+} sites and to a third site known to be present on E_1; this stabilizes E_1 at the expense of E_2 when [Mg^{2+}] is large. Stabilization of E_1 at pH 6.2 and 25 °C was found to be a highly cooperative function of [Mg^{2+}] and was not prevented by increasing [P_i]. The latter result requires the existence of a binding site for P_i on E_1, with an affinity for P_i comparable to that of E_2. Cooperativity with respect to [Mg^{2+}] requires that E_2 is the stable state of the enzyme in the absence of ligands, with an equilibrium constant [E_2]/[E_1] on the order of 10^3 or higher at pH 6.2 and 25 °C.

The Ca^{2+}-ATPase of skeletal muscle sarcoplasmic reticulum can exist in two major conformational states, E_1 and E_2 (de Meis & Vianna, 1979). E_1 has two high-affinity sites for Ca^{2+}, a high-affinity site for ATP, and possibly a second site of lower affinity for ATP. When both ATP and Ca^{2+} are bound, E_1 is converted to an ADP-sensitive phosphoenzyme, Ca_2E_1–P. E_2 binds one Mg^{2+} and one inorganic orthophosphate (P_i)[1] ion and, when both are bound, can be converted to an ADP-insensitive phosphoenzyme, MgE_2–P.

In a previous paper (Martin & Tanford, 1981), we studied the thermodynamics of formation of MgE_2–P from unliganded enzyme (E_1 + E_2). At low Mg^{2+} concentration (to about 10 mM), the extent of phosphorylation was found to obey the simple bireactant scheme shown in the right half of Figure 1, and our results and derived equilibrium constants were found to be in good agreement with the previous data of Punzengruber et al. (1978). At high [Mg^{2+}], however, the extent of phosphorylation was found to decrease, in qualitative agreement with earlier observations by Kanazawa (1975) and de Meis (1976). We suggested that the simplest way to account for this result is in terms of the stabilization of the E_1 form of the enzyme by high [Mg^{2+}]. The principal purpose of the present paper is to examine this possibility in more detail.

It is known that the formation of MgE_2–P is suppressed by the presence of even low levels of Ca^{2+}, owing to the stabilization of the E_1 state by the formation of Ca_2E_1. It is also known, however, that Mg^{2+} can compete with weak affinity for the Ca^{2+} binding sites (Yamada & Tonomura, 1972; Kalbitzer et al., 1978), and there is good evidence (Dupont, 1980; Kalbitzer et al., 1978) that there is a third binding site for divalent cations on E_1, which has a higher affinity for Mg^{2+} than for Ca^{2+}. There is thus the possibility for formation of complexes between Mg^{2+} and E_1 up to a level of Mg_3E_1 which can suppress MgE_2–P formation in the absence of Ca^{2+} by the same thermodynamic principle by which low levels of added Ca^{2+} lead to suppression. The equilibria involved in this mechanism are shown in the left half of Figure 1. Binding of Mg^{2+} to E_1 is expressed in the figure in terms of the suc-

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[1] Abbreviations used: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; P_i, inorganic phosphate (ortho); Tris, tris(hydroxymethyl)aminomethane.

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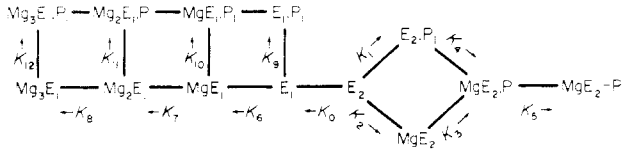

FIGURE 1: Minimal reaction scheme required to account for the experimental results. The arrows indicate the directions of each reaction step used for definition of the corresponding K; e.g., $K_0 = [E_1]/[E_2]$, $K_6 = [MgE_1]/([E_1][Mg^{2+}])$, and $K_9 = [E_1{\cdot}P_i]/([E_1][P_i])$. The equilibrium constants relating the P_i-containing forms of E_1 are not independent, so that no symbols for them are required.

cessive binding constants K_6–K_8. If we assume that the two high-affinity Ca^{2+} binding sites are identical and independent, with an intrinsic association constant K_α for Mg^{2+}, and if we assume K_β is the association constant for the third site mentioned above, the relation between K_6–K_8 and K_α and K_β is given by the following equations:

$$K_6 = 2K_\alpha + K_\beta \quad (1)$$

$$K_6K_7 = K_\alpha^2 + 2K_\alpha K_\beta \quad (2)$$

$$K_6K_7K_8 = K_\alpha^2 K_\beta \quad (3)$$

Since E_2 is the form of the enzyme with an established binding site for P_i, it was anticipated that an increase in the free phosphate concentration might counteract the inhibitory effect of high $[Mg^{2+}]$ by stabilizing E_2 at the expense of E_1. This possible effect was not observed, and this requires introduction of a binding site for P_i on E_1, as expressed by the species $Mg_3E_1{\cdot}P_i$, $Mg_2E_1{\cdot}P_i$, $MgE_1{\cdot}P_i$, and $E_1{\cdot}P_i$ in Figure 1. There are previous indications of association between P_i and E_1 from the work of Pucell & Martonosi (1971) and Hasselbach & Migala (1977).

Materials and Methods

Equilibrium levels of phosphoenzyme were measured at pH 6.2, 25 °C, in the presence of 5 mM EGTA, essentially as previously described (Martin & Tanford, 1981). Purified sarcoplasmic reticulum vesicles (0.9 mg/mL protein) were incubated with $^{32}P_i$ for 10 s, the reaction was quenched by addition of trichloroacetic acid and unlabeled H_3PO_4, and the precipitated protein was pelleted by centrifugation. Phosphorylation was carried out in 50 mM histidine–Tris buffer (pH 6.2) in place of the Mes–Tris buffer used previously because histidine and Tris have a lower affinity for Mg^{2+} than Mes (Albert, 1952; Good et al., 1966). ATPase activity was determined by using the coupled enzyme assay previously described (Dean & Tanford, 1977).

As was noted in the previous paper, complex formation between Mg^{2+} and inorganic phosphate or EGTA leads to the necessity of correcting added reagent concentrations if specific values for free concentrations of Mg^{2+} and P_i are desired. The correction was made, as before, by using the association constants listed by Vianna (1975) and Punzengruber et al. (1978). Because of the high $[Mg^{2+}]$ employed in many of the solutions, complexed phosphate constitutes a much larger fraction of total added phosphate than in the previous study (>50% when $[Mg^{2+}] > 75$ mM), and this required a change in the experimental procedure. It was desired to measure phosphoenzyme formation as a function of the Mg^{2+} concentration at *constant* free $[P_i]$, and the amount of P_i to be added to each sample solution had to be precalculated to achieve this goal. Total added P_i concentration ranged from 1 to 18 mM (free P_i 1–5 mM), and total added Mg^{2+} concentration ranged from 5 to 215 mM (free Mg^{2+} 5–200 mM). In the subsequent presentation of data, $[Mg^{2+}]$ and $[P_i]$ will always be used to refer to the *free* concentrations.

All samples were assayed in triplicate and were corrected for "nonspecific" ^{32}P incorporation and for loss of phosphoenzyme during washing as previously described. Nonspecific ^{32}P incorporation was proportional to total added P_i at low P_i concentrations, but a nonlinear increase was observed when added P_i reached the high levels required to maintain constant free P_i in the presence of high levels of Mg^{2+}. At 200 mM Mg^{2+}, as much as 40% of the observed radioactivity was estimated as being nonspecific. This means that the results at this Mg^{2+} concentration are subject to greater experimental error than data at $[Mg^{2+}] \leq 150$ mM.

Another consequence of extending the experimental measurements to high $[Mg^{2+}]$ is systematic variation in ionic strength in each series of experiments, from 0.1 at low $[Mg^{2+}]$ to as high as 0.9 at high $[Mg^{2+}]$, both values being in molar units. Punzengruber et al. (1978) have shown that ionic strength per se has no effect on phosphoenzyme formation. We have assumed that likewise it has no significant influence on the formation of complexes with the E_1 form of the ATPase.

Least-Squares Analysis of Equation 4. Values for a_0 through a_3 at a single value of $[P_i]$ were obtained by fitting the $[E_t]/[MgE_2{-}P]$ data obtained as a function of $[Mg^{2+}]$ directly to eq 4. The data at the various P_i and Mg^{2+} concentrations were pooled and fitted to eq 4 and 5 to obtain least-squares estimates of coefficients b_j and c_j. A direct-search algorithm (Chandler, 1976) was used to obtain the values of the coefficients which minimize the sum of the squared residuals, i.e., $\sum(y_{calcd} - y_{obsd})^2$, where $y = [E_t]/[MgE_2{-}P]$, and the summation extends over all observations, except that the data at 200 mM Mg^{2+} were omitted because, as noted above, they are less accurate than the data at lower Mg^{2+} concentrations. (As Figure 2 will show, two of the four measurements at 200 mM Mg^{2+} fall on the calculated curves based on the data at lower Mg^{2+} concentrations, and the other two deviate significantly.) The errors in the fitted parameters were established by using the method of support planes (Bevington, 1969) and represent one standard deviation.

The values of the fitted parameters were not dependent on where in parameter space the search was started. Since negative values of b_j and c_j are physically meaningless, these parameters were constrained to be greater than or equal to zero. This constraint did not have a statistically significant effect on the overall fit. More importantly, it was found that the parameters which went to zero did not have a significant influence on the remaining parameters.

Results

Phosphoenzyme formation was measured at each of four phosphate concentrations over the range of 5–200 mM free Mg^{2+}. The results are shown in Figure 2. A few isolated measurements made at lower $[P_i]$ are not shown. The dashed line shows the predicted dependence of phosphoenzyme level on $[Mg^{2+}]$, at one phosphate concentration, obtained by using the parameters of the preceding paper (Martin & Tanford, 1981). These parameters were based on data obtained at $[Mg^{2+}] \leq 12$ mM and are seen to fit the present data up to $[Mg^{2+}] = 25$ mM. The decrease in phosphoenzyme level at higher $[Mg^{2+}]$ is the effect with which this paper is concerned and which we shall demonstrate can be accounted for by the reaction scheme shown in Figure 1. The solid lines in Figure 2 are calculated curves based on the parameters derived from the data as described below.

Table I shows that the changes in the phosphoenzyme level in response to changes in $[P_i]$ and $[Mg^{2+}]$, including the decrease at high $[Mg^{2+}]$, are reversible. Each experiment was

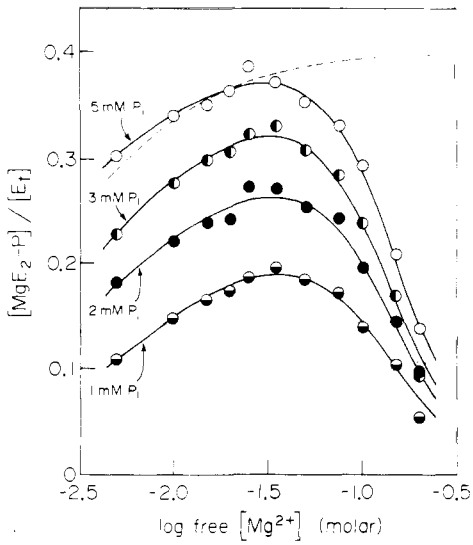

FIGURE 2: Equilibrium phosphoenzyme level for purified Ca^{2+}-ATPase vesicles (0.9 mg/mL protein) at pH 6.2, 25 °C. Each series of experiments was conducted at constant free $[P_i]$. The dashed line at 5 mM P_i is calculated from the equilibrium parameters of Martin & Tanford (1981). The solid lines are calculated from the a_j coefficients in the lower half of Table II.

Table I: Reversibility of Inhibition by Mg^{2+}

$[P_i]$ (mM)	$[Mg^{2+}]$ (mM)	$[MgE_2\text{-}P]/[E_t]$ exptl	$[MgE_2\text{-}P]/[E_t]$ calcd[a]
Initial Conditions (1.8 mg/mL Protein)			
5.8	200	0.174	0.157
Conditions after Dilution (0.3 mg/mL Protein)			
0.9[b]	200	0.061	0.064
2.3[c]	200	0.106	0.112
2.3[d]	33	0.284	0.284

[a] Calculation based on the parameters that describe the data of Figure 2. [b] Diluting buffer contained 200 mM Mg^{2+} and no P_i. [c] Diluting buffer contained 200 mM Mg^{2+} and 6.2 mM total P_i. [d] Diluting buffer contained neither Mg^{2+} nor P_i.

begun by preparation of a solution of 5.8 mM P_i and 200 mM Mg^{2+} and at relatively high protein concentration. The phosphoenzyme level in this solution was consistent with the data of Figure 2. Dilution with Mg^{2+}-containing buffer, so as to keep $[Mg^{2+}]$ constant, led to the expected decrease in phosphoenzyme level as a result of the decrease in $[P_i]$. Dilution with buffer alone, which decreases both $[P_i]$ and $[Mg^{2+}]$ (not to the same extent, because of the effect of Mg–P_i complexes on the concentration of the uncomplexed species), led to the expected *increase* in phosphoenzyme level.

In addition to the experiments of Table I, the absence of an irreversible effect of high $[Mg^{2+}]$ on the ATPase was also demonstrated by dilution of the enzyme, which had been preincubated with 200 mM Mg^{2+} and 5 mM P_i, into the standard medium for assay of ATPase activity. Full activity was found to have been retained.

The reversibility of changes in the phosphoenzyme level demonstrates that the data of Figure 2 represent equilibrium data, and we have analyzed them according to the equilibria presented in the scheme of Figure 1. The relation between phosphoenzyme level and reactant concentrations, according to this scheme, is most conveniently written in double-reciprocal form. For each series of experiments at *constant* $[P_i]$

$$\frac{[E_t]}{[MgE_2\text{–}P]} = a_0 + \frac{a_1}{[Mg^{2+}]} + a_2[Mg^{2+}] + a_3[Mg^{2+}]^2 \quad (4)$$

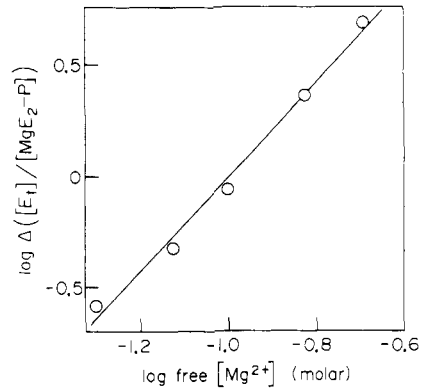

FIGURE 3: Double-logarithmic plot of the Mg^{2+} dependence of the difference between *observed* values of $[E_t]/[MgE_2\text{–}P]$ at 5 mM P_i and *predicted* values based on the dashed line of Figure 2. The line drawn is the least-squares linear regression line and has a slope of 2.13 ± 0.15.

where $[E_t]$ is the total concentration of ATPase in all states, based as before on a polypeptide chain molecular weight of 119000, which is equivalent to setting the parameter n of the preceding paper (Martin & Tanford, 1981) equal to unity. The coefficients a_j of this equation each contain one term which is the same at all phosphate concentrations and one term proportional to $1/[P_i]$, which can be represented by

$$a_j = b_j + c_j/[P_i] \quad (5)$$

Alternatively, this may be written as

$$a_j[P_i] = c_j + b_j[P_i] \quad (6)$$

The coefficients b_j and c_j are functions of the equilibrium constants of the scheme of Figure 1, as given by the following relations:

$$b_0 = (1 + K_5)/K_5 + K_0K_6K_{10}/(K_2K_3K_5) \quad (7)$$

$$c_0 = 1/(K_3K_5) + K_0K_6/(K_2K_3K_5) \quad (8)$$

$$b_1 = 1/(K_4K_5) + K_0K_9/(K_2K_3K_5) \quad (9)$$

$$c_1 = (1 + K_0)/(K_2K_3K_5) \quad (10)$$

$$b_2/K_{11} = c_2 = K_0K_6K_7/(K_2K_3K_5) \quad (11)$$

$$b_3/K_{12} = c_3 = K_0K_6K_7K_8/(K_2K_3K_5) \quad (12)$$

The parameters that focus exclusively on the Mg^{2+} binding equilibria of state E_1 are

$$c_2/c_1 = K_0K_6K_7/(1 + K_0) \quad (13)$$

$$c_3/c_1 = K_0K_6K_7K_8/(1 + K_0) \quad (14)$$

Since K_6, K_7, and K_8 are related by eq 1–3 to the two intrinsic binding constants K_α and K_β, there are three independent equilibrium constants on the right-hand sides of eq 13 and 14. With only two experimental parameters, unique values for these equilibrium constants obviously cannot be obtained. The analysis will show that narrow limits can be assigned to K_0 and to the product $K_\alpha^2K_\beta$.

A variety of procedures can be used to obtain the coefficients of eq 4 from the curves of Figure 2. All show that the descending limbs of the curves at high $[Mg^{2+}]$ are highly cooperative with respect to the concentration of Mg^{2+}; i.e., the term $a_3[Mg^{2+}]^2$ is the dominant term, and a_2 must be close to zero. (It should be noted that this proves the involvement of three binding sites for Mg^{2+} on E_1.) A simple demonstration of the cooperativity is provided by the data on Figure 3, which are derived from the results of Figure 2 at 5 mM P_i and $[Mg^{2+}] \geq 50$ mM. The figure shows a double logarithmic plot

Table II: Coefficients of Equation 4

$[P_i]$ (mM)	a_0	$10^2 a_1$	a_2	a_3
	Unrestricted Analysis			
1	4.22 ± 0.26	2.49 ± 0.17	3 ± 7	215 ± 38
2	3.56 ± 0.26	1.01 ± 0.16	−7 ± 7	195 ± 37
3	2.82 ± 0.09	0.80 ± 0.06	−3 ± 2	157 ± 13
5	2.67 ± 0.07	0.33 ± 0.04	−5 ± 2	130 ± 10
	With a_2 Set Equal to Zero			
1	4.32 ± 0.10	2.44 ± 0.10	0	231 ± 8
2	3.32 ± 0.10	1.15 ± 0.11	0	158 ± 9
3	2.70 ± 0.04	0.86 ± 0.04	0	139 ± 3
5	2.48 ± 0.04	0.43 ± 0.04	0	100 ± 4

of the *difference* between the observed values of $[E_t]/[MgE_2\text{–}P]$ and the corresponding values predicted by the dashed line of Figure 2, plotted as a function of $[Mg^{2+}]$. This difference results from the contributions of the last two terms of eq 4, and the plot has a slope close to 2.0, as expected from the final term alone. Cooperativity of this kind implies that an unfavorable conformational change of the protein is required before the inhibitory effect of Mg^{2+} can be exerted (Monod et al., 1965) and suggests that the equilibrium constant $K_0 = [E_1]/[E_2]$ in the scheme of Figure 1 must be small. The subsequent analysis will confirm this.

The most objective way to obtain numerical values for the coefficients of eq 4 is to perform a least-squares analysis on the data as described under Materials and Methods. Results of this analysis, carried out independently for each data set at constant $[P_i]$, are shown in Table II. The values of a_0 that were obtained are virtually identical with the values predicted from the data at low magnesium concentrations and at 25 °C reported earlier (Martin & Tanford, 1981). The values of a_1 agree less well with the earlier data (mean deviation about 25%), which is perhaps not surprising because the term in eq 4 ($a_1/[Mg^{2+}]$) makes it largest contribution at $[Mg^{2+}] < 5$ mM, i.e., outside the concentration range of the present study. Repetition of the analysis with assigned values of a_1 (based on the parameters of the preceding paper) did not significantly alter the values of a_0, a_2, and a_3.

The most striking result of the analysis is that the coefficient a_2 is within experimental error equal to zero. Since negative values would be physically meaningless, one can only say that a_2 must be much smaller than a_3 at each phosphate concentration. The lower half of Table II is the result obtained when the analysis is carried out with a_2 fixed at zero. This was found to have no statistically significant effect on the quality of the overall fit. The values for a_3 differ somewhat from those in the upper half of the table, but more importantly, the error in a_3 decreases markedly, as is expected if the nonzero values of a_2 in the upper half reflect experimental scatter of the data.

Table II shows that the coefficient a_3 is strongly dependent on $[P_i]$. This means that one cannot fit the experimental data without allowing for the ability of Mg_3E_1 to bind P_i. (Presumably, all forms of E_1 have binding sites for P_i, as indicated in the scheme of Figure 1, but the cooperativity of the reaction with respect to $[Mg^{2+}]$ makes it impossible to obtain direct experimental data for any form of E_1 other than Mg_3E_1.) Either eq 5 or eq 6 can be used to analyze the dependence of a_3 on $[P_i]$ to give $K_{12} = b_3/c_3$. The two equations give similar, but not identical results (because $1/[P_i]$ is not a linear function of $[P_i]$), and the combined result is $K_{12} = 450 \pm 150$ M^{-1}. This result shows that the E_1 form of the enzyme is capable of binding P_i with about the same affinity as E_2. With K_0 small (which this paper demonstrates), the equilibrium constants of the preceding paper (Martin & Tanford, 1981) represent the true equilibria of E_2. The equilibrium constants (K_1 and K_3) obtained at 25 °C were 105 and 524 M^{-1}, respectively, for the reactions $E_2 + P_i \rightleftharpoons E_2{\cdot}P_i$ and $MgE_2 + P_i \rightleftharpoons MgE_2{\cdot}P_i$.

The affinity of E_1 for P_i is not a major concern of this paper, the main focus being on the equilibria for binding of Mg^{2+}. Analysis of these equilibria requires values for c_1, c_2, and c_3. Combining eq 2, 3, 13, and 14, we have

$$c_3/c_1 = K_0 K_\alpha^2 K_\beta/(1 + K_0) \quad (15)$$

$$c_2/c_3 = 1/K_8 = 1/K_\beta + 2/K_\alpha \quad (16)$$

We can obtain c_3 from the a_3 values of Table II by use of eq 5 or 6 (as described in the preceding paragraph), and this gives a value for c_3/c_1 of about 1×10^4 M^{-3} (Table III). The a_2 values of Table II do not, however, permit evaluation of c_2. One can only say that c_2 (and hence c_2/c_3) is approximately zero, which leads by eq 15 and 16 to $K_0 = 0$ and $K_\alpha^2 K_\beta = \infty$.

A positive value for c_2 can be obtained by enlarging the data pool for the least-squares analysis. All of the data of Figure 2 were analyzed together, and some additional points at lower $[P_i]$ (data not shown) were included. All eight b_j and c_j parameters of eq 7–12 were evaluated, and the pertinent c_j values are shown in Table III. A formally positive value for c_2 was obtained, from which, by standard propagation of error analysis (Bevington, 1969), the most probable value of c_2/c_3 is 0.022 M. The analysis places an upper limit of 0.062 M on c_2/c_3; the lower limit is still zero. Table IV shows mutually compatible values of K_0, K_α, and K_β, based on eq 15 and 16. The values of K_0 that correspond to any given value of c_2/c_3 depend on the relative magnitudes of K_α and K_β, the largest possible value of K_0 being obtained when $K_\alpha = K_\beta$.

Discussion

The principal purpose of this paper has been to show that the inhibitory effect of high $[Mg^{2+}]$ on the phosphorylation of the Ca^{2+}-ATPase by inorganic phosphate can be explained by the binding of Mg^{2+} to the E_1 form of the enzyme. Satisfactory fit of the data was obtained by use of the scheme of Figure 1, as is illustrated by the theoretical curves of Figure 2. These curves were obtained by using eq 4, with the coefficients listed in the lower half of Table II. The data do not lend themselves to an accurate determination of the equilibrium constants for binding of Mg^{2+} to the E_1 form, but the data unambiguously require three binding sites, in agreement with the conclusion of Dupont (1980) that three sites for divalent metal binding are involved in the activation of ATP hydrolysis. Since activation occurs via the E_1 form of the enzyme, binding sites that promote activation must reside on the E_1 form. Recent data of Kalbitzer et al. (1978) also suggest three binding sites on E_1. They studied Mg^{2+} binding to sarcoplasmic reticulum vesicles by a method involving competition between Mg^{2+} and Mn^{2+} for the same sites and observed three principal independent binding sites attributable to the Ca^{2+}-ATPase. Two of the sites are the sites to which Ca^{2+} binds with high affinity and, therefore, represent sites on the E_1 form of the enzyme. The third site can also be assumed to represent a site on E_1 because the observed binding constant is nearly 2 orders of magnitude higher than the accurately determined equilibrium constant for the binding of Mg^{2+} to E_2 (Punzengruber et al., 1978; Martin & Tanford, 1981).

It may be noted in this connection that the existence of unambiguous K_α and K_β values would make it possible to evaluate K_0 from c_3/c_1 alone by use of eq 15. The numerical results of Kalbitzer et al. (1978) cannot unfortunately be used

Table III: Coefficients Related to Mg^{2+} Binding[a]

	graphical anal of data in Table II	least-squares anal of combined data
c_1	$(1.6 \pm 0.3) \times 10^{-5}$	
c_2	0	$(3.2 \pm 5.9) \times 10^{-3}$
c_3	0.17 ± 0.03	0.15 ± 0.03
c_3/c_1	$(1.1 \pm 0.3) \times 10^4$	$(0.93 \pm 0.27) \times 10^4$
c_2/c_3	0	0.022 ± 0.040

[a] The value of c_1 derived from the data of the previous paper was used, being more reliable than the value obtained from the present work (see text). This has no effect on the conclusion of the paper.

Table IV: Compatible Values for K_0 and Mg^{2+} Binding Constants[a]

$c_2/c_3 = K_s^{-1}$ (M)	assumed K_β/K_α	K_0	K_α (M^{-1})	K_β (M^{-1})
0.062[b]	1	0.09	48	48
	10	0.024	34	340
	50	0.005	33	1600
0.022[c]	1	0.004	140	140
	10	0.0011	95	950
	50	2×10^{-4}	92	4500
0.0089[d]	(26)	3×10^{-5}	230	6000
0[e]		0	$K_\alpha^2 K_\beta = 00$	

[a] For each value of c_2/c_3, $K_\beta = K_\alpha$ gives the largest possible value for K_0. Assuming $K_\beta/K_\alpha < 1$ has qualitatively the same effect as assuming $K_\beta/K_\alpha > 1$. [b] Statistical upper limit. [c] Statistically most probable value. [d] Data derived from the Mg^{2+} binding constants at pH 6.8 given by Kalbitzer et al. (1978), without use of a c_2/c_3 value based on the present data. [e] The data of this paper do not establish a lower limit for c_2/c_3 other than the limit imposed by the condition that c_2/c_3 (i.e., $1/K_s$) has to be positive.

for this purpose because they did not include the $E_1 \rightleftharpoons E_2$ equilibrium in their analysis; i.e., their equilibrium constants may not be directly comparable to the K_α and K_β values defined in this paper. As Table IV shows, however, use of their binding constants as representative of K_α and K_β does not lead to any inconsistency with the results of this paper.

An unanticipated result of our study is the dependence of Mg^{2+} binding to E_1 on the concentration of inorganic phosphate. This requires the existence of a binding site for P_i in the E_1 state of the enzyme, as expressed by the binding constants K_9–K_{12} in the scheme of Figure 1. The data permitted quantitative estimation of only one of these constants ($K_{12} \simeq 500\ M^{-1}$), which proved to be of the same order of magnitude as the equilibrium constants for binding to the E_2 form. It is reasonable to suppose that the phosphate binding site demonstrated in this way is a site that binds ATP or other nucleoside phosphates in the normal operation of the pump. Competition between P_i and nucleoside phosphates for the same site, or possibly for two sites of different affinities, has been suggested by Pucell & Martonosi (1971) and by Hasselbach & Migala (1977). Our data on the stabilization of Mg_3E_1 by P_i are not sufficient to distinguish whether one or two sites are involved.[2]

The most important aspect of our data is the high degree of cooperativity of the inhibitory effect of Mg^{2+}, as demonstrated by Figure 3 and by the low values for c_2 in Table III. This result requires that K_0 be much less than 1, most likely on the order of 10^{-3} or less. The data of Table IV show that an upper limit of $K_0 = 0.09$ would be consistent with our data if one could assume $K_\alpha = K_\beta$. The results of Kalbitzer et al. (1978) give $K_\beta/K_\alpha = 26$. The fact that their results were derived from an analytical treatment that ignored the $E_1 \rightleftharpoons E_2$ equilibrium is likely to have less effect on this ratio than on the individual values of K_α and K_β. The upper limit of K_0 in Table IV is therefore probably too high. Regardless of this upper limit, it is clear that the ligand-free ATPase exists under the conditions of our experiments (pH 6.2, 25 °C) predominantly in the E_2 form, with $[E_2]/[E_1]$ probably $\sim 10^3$ or higher.

It is of interest in this connection that the most recent (and perhaps most accurate) study of Ca^{2+} binding to unliganded ATPase (Inesi et al., 1980) also showed a high degree of cooperativity with a Hill plot slope of 1.82 (at pH 6.8). Alternatively, these results could be expressed in terms of successive binding constants $K_{1'}$ and $K_{2'}$ with $K_{2'}/K_{1'} = 400$. If the cooperativity is interpreted in terms of the standard model (Monod et al., 1965) for allosteric effects (i.e., $Ca_2E_1 \rightleftharpoons E_1 \rightleftharpoons E_2$, with two identical and independent Ca^{2+} binding sites on E_1), one obtains $K_0 = 6 \times 10^{-4}$ (or $1/K_0 = 1.5 \times 10^3$), which is similar to the estimates based on our Mg^{2+} inhibition data.

An additional conclusion that follows from the necessarily small value of K_0 is that the terms containing K_0 in eq 7–10 all make a negligible contribution to the coefficients b_0, c_0, b_1, and c_1 even if the least favorable choice is made for the equilibrium constants for which a range of values is possible. This means that the *apparent* values of K_1–K_5 given in our previous paper (Martin & Tanford, 1981) and by Punzengruber et al. (1978) can be regarded as the true equilibrium constants for the phosphorylation sequence of E_2. Even with $K_0 = 0.09$ (top line of Table IV), the largest difference between the true binding constants for E_2 and the experimental binding constants reported in the previous paper would be 10%.

[2] Figure 1 does not explicitly allow for the possibility that the protein may have binding sites that can bind $Mg{\cdot}P_i$ as a unit, instead of binding Mg^{2+} or P_i independently. Species that would result from this type of association are in fact *implicitly* included in the analysis, because they would contribute to already existing terms in eq 4–6 and their presence would be reflected in the numerical values of the coefficients of these equations. The actual results eliminate the possibility that $Mg{\cdot}P_i$ binding is an important factor. This is perhaps intuitively evident from the raw results of Figure 2. Although the ratio $[Mg{\cdot}P_i]/[Mg^{2+}]$ increases as $[P_i]$ increases, the concentration of Mg^{2+} at which inhibition first sets in is independent of $[P_i]$. The numerical results confirm this conclusion. For example, if the P_i site on E_1 and the β site for Mg^{2+} were a single site that preferentially binds to $Mg{\cdot}P_i$, it would be reflected in a much larger value for K_{12} than was observed. Similarly, if $Mg{\cdot}P_i$ could compete for the P_i site on E_2, and if that contributed significantly to the inhibition, it would lead to a large value for the coefficient a_2 instead of the near-zero value obtained experimentally.

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Determination of the Free-Energy Coupling for Binding of Calcium Ions and Troponin I to Calmodulin†

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ABSTRACT: Regulation of a wide variety of biological systems by Ca^{2+} is now known to be mediated through calmodulin, a Ca^{2+}-binding protein. Calmodulin forms Ca^{2+}-dependent complexes with several proteins, including troponin I. We have determined the free-energy coupling ($\Delta G°_{CT}$) for binding of Ca^{2+} and troponin I to calmodulin by measuring Ca^{2+} binding to calmodulin and to the 1:1 calmodulin·troponin I complex by equilibrium dialysis. The dissociation constant for the $Ca^{2+}{}_4$·calmodulin and troponin I complex was also determined directly by monitoring fluorescence intensity changes accompanying complex formation between $Ca^{2+}{}_4$·CaM and *N*-dansylaziridine–troponin I, a fluorescent derivative of troponin I. Calmodulin displayed four Ca^{2+}-binding sites of similar affinity with a geometric mean dissociation constant of 14 μM. In the presence of saturating troponin I, the geometric mean dissociation constant for the four Ca^{2+}-binding sites was shifted to 1.7 μM. $\Delta G°_{CT}$ was therefore −1.25 kcal/mol of Ca^{2+}. Saturation of calmodulin with Ca^{2+} would therefore be expected to increase its affinity for troponin I about 4500-fold. A dissociation constant of 20 nM was determined for the $Ca^{2+}{}_4$·calmodulin·*N*-dansylaziridine–troponin I complex. The dissociation constant of the calmodulin·*N*-dansylaziridine–troponin I complex in the absence of Ca^{2+} should therefore be about 90 μM. A prediction of this data is that binding of Ca^{2+} to calmodulin will show strong positive cooperativity when Ca^{2+} binding to a substoichiometric number of sites is sufficient to promote calmodulin·troponin I complex formation.

Calmodulin (CaM)[1] was discovered by Cheung as an activator of the Ca^{2+}-sensitive 3′:5′-cyclic-nucleotide phosphodiesterase (Cheung, 1970). This regulatory protein mediates Ca^{2+} stimulation of several enzymes including an isozyme of 3′:5′-cyclic-nucleotide phosphodiesterase (Cheung, 1970; Kakiuchi et al., 1970), brain adenylate cyclase (Brostrom et al., 1975), myosin light chain kinase (Dabrowska et al., 1978), phosphorylase kinase (Cohen et al., 1978), (Ca^{2+} + Mg^{2+})-ATPase (Gopinath & Vincenzi, 1977; Jarrett & Penniston, 1977), NAD kinase (Anderson & Cormier, 1978), and phospholipase A_2 (Wong & Cheung, 1979). CaM also mediates the Ca^{2+} sensitivity of microtubule disassembly and contraction during mitosis (Welsh et al., 1978) and binds in a Ca^{2+}-dependent manner to troponin I (Amphlett et al., 1976) and to calcineurin (Wang & Desai, 1977; Klee & Krinks, 1978). Ca^{2+}-dependent complex formation between CaM and several of these proteins has been demonstrated by a number of techniques including gel filtration (Teshima & Kakiuchi, 1974), electrophoresis on nondenaturing gels (Amphlett et al., 1976; LaPorte & Storm, 1978), CaM–Sepharose affinity chromatography (Watterson & Vanaman, 1976; Klee & Krinks, 1978; Westcott et al., 1979), fluorescence techniques (LaPorte et al., 1981), and cross-linking of [^{125}I]CaM to CaM-binding proteins LaPorte et al., 1979; Richman & Klee, 1978; Andreasen et al., 1981).

Although it is generally agreed that CaM binds 4 mol of Ca^{2+}, there are significant differences in the reported affinities for Ca^{2+} (Teo & Wang, 1973; Lin et al., 1974; Watterson et al., 1976; Wolff et al., 1977; Dedman et al., 1977; Jarrett & Kyte, 1979; Crouch & Klee, 1980). Some of these discrepancies in affinity of CaM for Ca^{2+} have been attributed to differences in ionic strength (Dedman et al., 1977). Binding of Ca^{2+} to CaM in the presence of CaM-binding proteins has not been well-characterized. A widely accepted model for interaction of CaM and the Ca^{2+}-sensitive phosphodiesterase is based on results from several laboratories [reviewed in Wang (1977)]:

$$n\mathrm{Ca}^{2+} + \mathrm{CaM} \rightleftarrows \mathrm{Ca}^{2+}{}_n\cdot\mathrm{CaM}^*$$

$$(\mathrm{PDE})_2 + 2(\mathrm{Ca}^{2+}{}_n\mathrm{CaM}^*) \rightleftarrows (\mathrm{Ca}^{2+}{}_n\cdot\mathrm{CaM}^*)_2(\mathrm{PDE})_2 \rightleftarrows (\mathrm{Ca}^{2+}{}_n\cdot\mathrm{CaM}^*)_2(\mathrm{PDE}^*)_2$$

where PDE represents one subunit of the dimeric phospho-

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[1] Abbreviations: AEDANS-CaM, 5-[[(acetylamino)ethyl]amino]-1-naphthylaminesulfonic acid labeled calmodulin; CaM, calmodulin; DANZ-TnI, *N*-dansylaziridine-labeled troponin I; DNP-Gly, dinitrophenylglycine; DTT, dithiothreitol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; [^{125}I]CaM, ^{125}I-labeled calmodulin; $\bar{K}_d$, geometric mean dissociation constant; Mops, 3-(*N*-morpholino)propanesulfonic acid; PDE, Ca^{2+}-sensitive 3′:5′-cyclic-nucleotide phosphodiesterase; TnI, troponin I; EPPS, 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid.

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