Biochimica et Biophysica Acta, 298 (1973) 906–926

BBA 76271

ATP AND Ca^{2+} BINDING BY THE Ca^{2+} PUMP PROTEIN OF SARCOPLASMIC RETICULUM

GERHARD MEISSNER

Department of Molecular Biology, Vanderbilt University, Nashville, Tenn. 37235 (U.S.A.)

(Received October 17th, 1972)

SUMMARY

The binding of ATP and Ca^{2+} by the Ca^{2+} pump protein of sarcoplasmic reticulum from rabbit skeletal muscle has been studied and correlated with the formation of a phosphorylated intermediate. The Ca^{2+} pump protein has been found to contain one specific ATP and two specific Ca^{2+} binding sites per phosphorylation site. ATP binding is dependent on Mg^{2+} and is severely decreased when a phosphorylated intermediate is formed by the addition of Ca^{2+}. In the presence of Mg^{2+} and the absence of Ca^{2+}, ATP and ADP bind competitively to the membrane. Preincubation with *N*-ethylmaleimide results in inhibition of ATP binding and decrease of Ca^{2+} binding. In the absence of ATP, Ca^{2+} binding is noncooperative at pH 6–7 and negatively cooperative at pH 8. Mg^{2+}, Sr^{2+} and La^{3+}, in that order, decrease Ca^{2+} binding by the Ca^{2+} pump protein. The affinity of the Ca^{2+} pump protein for both ATP and Ca^{2+} increases when the pH is raised from 6 to 8. At the inflection point ($pH \approx 7.3$) the binding constants of the Ca^{2+} pump protein–$MgATP^{2-}$ and Ca^{2+} pump protein–calcium complexes are approx. 0.25 and 0.5 μM^{-1}, respectively. The unphosphorylated Ca^{2+} pump protein does not contain a Mg^{2+} binding site with an affinity comparable to those of the ATP and Ca^{2+} binding sites.

The affinity of the Ca^{2+} pump protein for Ca^{2+} is not appreciably changed by the addition of ATP. The ratio of phosphorylated intermediate formed to bound Ca^{2+} is close to 2 over a 5-fold range of phosphoenzyme concentration. The equilibrium constant for phosphoenzyme formation is less than one at saturating levels of Ca^{2+}. The phosphoenzyme is thus a "high-energy" intermediate, whose energy may then be used for the translocation of the two Ca^{2+}.

A reaction scheme is discussed showing that phosphorylation of sarcoplasmic reticulum proceeds *via* an enzyme–Ca_2^{2+}–$MgATP^{2-}$ complex. This complex is then converted to a phosphoenzyme intermediate which binds two Ca^{2+} and probably Mg^{2+}.

INTRODUCTION

Ca^{2+} uptake by sarcoplasmic reticulum vesicles is energized by ATP through a membrane-bound, Ca^{2+} stimulated ATPase. This ATPase activity involves several

Abbreviations: EGTA, ethyleneglycol-bis-(β-aminoethyl ether)-*N,N'*-tetraacetic acid; CHTA, (cyclohexylanedinitrilo)tetraacetic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

steps, including the presumable binding of Ca^{2+} and ATP, formation of a covalently linked phosphoenzyme intermediate, Ca^{2+} translocation and breakdown of the phosphorylated intermediate (*cf.* reviews[1,2]). These steps can be summarized as follows:

$$\text{Enzyme} + 2\,Ca^{2+}_{\text{outside}} + \text{ATP} \longrightarrow \text{enzyme} - P_{Ca_2^{2+}} \rightleftharpoons \text{enzyme} + P_i + 2\,Ca^{2+}_{\text{inside}}$$
$$\downarrow$$
$$\text{ADP}$$

The overall reaction results in the translocation of two Ca^{2+} from the outside to the inside of the vesicles and in the splitting of one molecule of ATP[3,4]. The above reaction may also occur in the reverse direction, resulting in the formation of ATP[5]. A ratio of two translocated Ca^{2+} to one hydrolyzed ATP implies that the Ca^{2+}-stimulated ATPase contains one ATP and 2 Ca^{2+}-binding sites per active site. From Ca^{2+} uptake experiments, ATPase activity and phosphoenzyme studies, it has been deduced that the binding constants for ATP and Ca^{2+} range from about 0.1 to 10 μM^{-1} (refs 1, 2, 6–8). Binding studies have shown that sarcoplasmic reticulum membranes bind ATP[3,9,10] and contain specific high-affinity Ca^{2+} binding sites with a binding constant of about 1 μM^{-1} (refs 11, 12). However, a clear and quantitative correlation between the number of ATP and Ca^{2+} binding sites and the number of phosphorylation sites has not been established.

In the present study we use a partially purified Ca^{2+} pump protein (ATPase) preparation[12] to investigate ATP and Ca^{2+} binding by sarcoplasmic reticulum under a variety of conditions. It was found that the Ca^{2+} pump protein contains one specific high-affinity ATP binding site and two specific high-affinity Ca^{2+} binding sites per active site. Further, a direct and quantitative correlation was made between ATP and Ca^{2+} binding and the formation of a phosphorylated intermediate.

METHODS AND MATERIALS

Preparations

Sarcoplasmic reticulum vesicles were prepared by zonal gradient centrifugation as previously described without using a salt wash (Fraction 2 of Table I of ref. 12). Partially purified Ca^{2+} pump protein (also referred to as ATPase protein or phosphoenzyme) was obtained by partially extracting sarcoplasmic reticulum vesicles with cholate or deoxycholate[12].

Assays

Ca^{2+} uptake, phosphoenzyme formation (at 0 °C), ATPase activity and protein and phosphorus determinations were carried out as previously described[12,13].

ATP binding

Unless otherwise indicated, the ATP binding assay was carried out with 1–3 mg sarcoplasmic reticulum protein in 4 ml of 100 mM KCl, 1.2 mM Mg^{2+}, 1 mM ethyleneglycol-bis-(β-aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA) and 10 mM histidine (pH 7.6). After incubation for 3 min at 0 °C, 2–40 μl of 1 mM [^{14}C]ATP (10^4–10^5 cpm/nmole ATP) were added with stirring. The suspension was pressed through a type VC 0.1-μm or GS 0.22-μm Millipore filter at 30 s and 1 min after

the addition of ATP. Type AP20 Millipore prefilters were used to increase the flow rate. It was curious that 0.1-μm Millipore filters were required when ATP binding by native sarcoplasmic reticulum vesicles was studied. The amount of [^{14}C]ATP bound was then determined by measuring the radioactivity of the solutions before and after filtration. When sarcoplasmic reticulum protein was omitted from the sample, less than 1% of the [^{14}C]ATP was bound by the Millipore filter apparatus and the filters. Each of the filters and prefilters contained 5–10 nmoles exchangeable Ca^{2+} which might have interfered with the ATP binding assay by causing phosphoenzyme formation. The Ca^{2+} can be largely removed by washing with 4 M HCl and 5 mM $MgCl_2$, rinsing with deionized water and subsequent drying in air. However, since the same degree of ATP binding was detected in experiments with washed and unwashed filters, unwashed filters were routinely used for the ATP binding assays.

Measurement of ATP hydrolysis in the binding assays

The amount of ATP hydrolyzed was determined by using [γ-^{32}P]ATP in the binding assays. 1 ml of the sample was added to 4 ml of 5% trichloroacetic acid containing 0.5 mM ATP and 1 mM P_i. After removal of 1 ml to determine total radioactivity, 0.2 g of Norit A (Fisher Scientific Co.) was added to the remaining 4 ml to adsorb nucleotides[14]. The suspension was shaken for 20 min at room temperature and the charcoal was then removed by filtration. The percent of total radioactivity remaining in the filtrate was set equal to the percent of ATP which was hydrolyzed to ADP and $^{32}P_i$.

Ca^{2+} binding

Unless otherwise indicated, Ca^{2+} binding was measured by incubating sarcoplasmic reticulum membranes for 45 min at 0 °C in 2 ml of a medium which contained 2 mg sarcoplasmic reticulum protein, 100 mM KCl, 5 mM $MgCl_2$, 10 mM Tris–histidine (pH 7.5) and various concentrations of $^{45}Ca^{2+}$. Aliquots were taken prior to centrifugation to determine total Ca^{2+} concentration by atomic absorption spectroscopy[12] and to measure total radioactivity. The membranous fraction was then removed by centrifugation for 1 h at 45000 rev./min in a Spinco No. 50 rotor and the radioactivity of the supernatant was measured to obtain the percent of $^{45}Ca^{2+}$ which was bound to the sarcoplasmic reticulum membranes.

Determination of radioactivity

100 μl of a sample containing either $^{45}Ca^{2+}$, [^{14}C]ATP, [^{14}C]ADP or [^{32}P]-ATP ($3 \cdot 10^2$–$3 \cdot 10^3$ cpm) were added to 4 ml of a scintillation fluid containing 60 g naphthalene, 4.2 g 2,5-diphenyloxazole, 180 mg 1,4-bis-[2-(5-phenyloxazolyl]benzene and 70 ml water in 900 ml dioxane. Counting was carried out in a Packard Tri-Carb liquid scintillation spectrometer, using mini vials and adapters (Nuclear Associates, Westbury, N.Y.).

Materials

$^{45}Ca^{2+}$, [8-^{14}C]ATP and [8-^{14}C]ADP were obtained from New England Nuclear (Boston, Mass.). ATP and ADP were purchased from P–L Biochemicals (Milwaukee, Wisc.). [γ-^{32}P]ATP was prepared according to Post and Sen[15] and was a generous gift of Dr Robert Post (Department of Physiology, Vanderbilt University).

RESULTS

ATP and ADP binding

The conditions of the ATP binding assay were chosen to minimize ATP hydrolysis. At 0 °C and in the presence of EGTA to complex small amounts of contaminating Ca^{2+}, less than 2% of the ATP was hydrolyzed within 5 min by Ca^{2+} pump protein. Larger amounts (up to about 10% within 1 min) were hydrolyzed when native sarcoplasmic reticulum vesicles were used in these studies.

Table I shows that the binding of ATP to both native sarcoplasmic reticulum vesicles and Ca^{2+} pump protein varies with the composition of the assay medium. At constant ATP and protein concentrations optimal binding is observed in a medium which contains an excess of Mg^{2+} and in which the free Ca^{2+} concentration is drastically lowered by addition of EGTA. Chelation of Mg^{2+} by EDTA or (cyclohexylanedinitrilo)tetraacetic acid (CHTA) results in a significant lowering of the amount of bound ATP suggesting that Mg^{2+} is required for optimal ATP binding to sarcoplasmic reticulum membranes. When the medium contains 0.1 mM Ca^{2+} and 5 mM Mg^{2+}, a reduction in bound ATP is also observed. Since these conditions are optimal for phosphoenzyme formation, the reaction is apparently proceeding beyond the binding stage. This is demonstrated by substituting [γ-^{32}P]ATP for [^{14}C]ATP, in which case 3.5 nmoles of ^{32}P were bound per mg protein. In the absence of free Ca^{2+} about equal amounts of [^{32}P]ATP and [^{14}C]ATP were bound by the Ca^{2+} pump protein. It should be noted that in these experiments the concentration of ATP is less than the concentration of phosphorylation sites.

When ATP binding by the Ca^{2+} pump protein was studied in the presence of 0.1 mM Ca^{2+}, 5 mM Mg^{2+} and either 10 μM [^{14}C]ATP or [^{32}P]ATP, 0.9 nmole of [^{14}C]ATP and 7.5 nmoles of ^{32}P per mg protein, respectively, were bound (not

TABLE I

ATP BINDING BY SARCOPLASMIC RETICULUM AND Ca^{2+} PUMP PROTEIN

The Ca^{2+} pump protein, prepared by partial extraction of sarcoplasmic reticulum vesicles with cholate, contained 12.8 μg bound P per mg protein and had a phosphoenzyme level of 7.9 nmoles ^{32}P per mg protein. Sarcoplasmic reticulum vesicles or Ca^{2+} pump protein were incubated in a solution containing 0.4 mg protein per ml, 2.4 μM [^{14}C]ATP or [γ-^{32}P] ATP, 100 mM KCl, 10 mM histidine (pH 7.6) and the indicated concentrations of Mg^{2+}, Ca^{2+}, EGTA, EDTA and CHTA.

Components added	*ATP binding (nmoles/mg protein)*		*^{32}P "binding" (nmoles/mg protein) Ca^{2+} pump protein*
	Sarcoplasmic reticulum	*Ca^{2+} pump protein*	
0.1 mM Ca^{2+}, 5 mM Mg^{2+}	0.5	0.5	3.5
1 mM EGTA, 5 mM EDTA	—	0.6	—
1 mM EGTA, 1 mM CHTA	0.7	0.7	0.5
0.1 mM EGTA, 0.125 mM Mg^{2+}	—	2.5	—
1 mM EGTA, 1.2 mM Mg^{2+}	2.3	2.7	2.5
1 mM EGTA, 5 mM Mg^{2+}	2.4	2.9	—

shown). Only about 15% of the ATP was hydrolyzed to ADP and P_i under these conditions. The low amount of [^{14}C]ATP bound indicates then that the ATP binding site is blocked in the phosphorylated intermediate. The ADP, which was formed in the reaction, could not have effectively competed with ATP for a prospective ATP binding site (*cf.* Fig. 3).

The binding of ATP to sarcoplasmic reticulum vesicles and the Ca^{2+} pump protein was analyzed in the absence of free Ca^{2+} (Fig. 1). Binding to the Ca^{2+} pump protein is dependent on the presence of Mg^{2+} over the entire range of ATP concentration tested. Scatchard plots of these data give straight lines with the same slope. Similar values are obtained when ATP binding by the Ca^{2+} pump protein is studied in the presence of 5 mM Mg^{2+} instead of 1.2 mM Mg^{2+} (not shown). Consequently, there is only one type of ATP binding site for these preparations in the presence of Mg^{2+} and over the ATP concentration tested. The binding constant, $K_{Mg\,ATP}$, calculated from the slope is $0.42 \cdot 10^6$ M^{-1} (or 0.42 μM^{-1}) (S.D. = ± 0.07 of five determinations). The intercepts on the abscissa yield 6.3 and 7.6 nmoles of binding sites per mg protein from sarcoplasmic reticulum vesicles and Ca^{2+} pump protein, respectively. The number of binding sites agree, within experimental error, with the phosphoenzyme levels of the sarcoplasmic reticulum vesicle and Ca^{2+} pump protein preparations. Thus there appears to be one ATP binding site per active site. It may be noted that the data were not corrected for "unspecific" ATP binding (*cf.* Table I), since this type of binding is only observed in the absence of Mg^{2+}. ATP^{4-} binding (in the absence of Mg^{2+}) was too weak to be analyzed without ambiguity by the Millipore filtration technique.

The binding constant of the ATP binding site is affected by the pH of the solution. In Fig. 2 the binding constant, $K_{Mg\,ATP}$, is plotted against the pH of the solution. $K_{Mg\,ATP}$ increases with increasing pH reaching a maximum value at pH 8.0

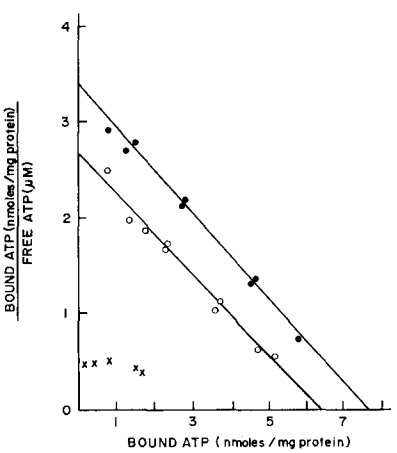


Fig. 1. Scatchard plots of ATP binding by sarcoplasmic reticulum vesicles and Ca^{2+} pump protein. Sarcoplasmic reticulum vesicles (○—○) and Ca^{2+} pump protein (●—● and ×—×) (12.8 μg bound P/mg protein), prepared by partial extraction of sarcoplasmic reticulum vesicles with cholate, had phosphoenzyme levels of 6.4 ± 0.3 and 7.9 ± 0.4 nmole ^{32}P per mg protein, respectively. The ATP binding assay was carried out with 0.25–0.75 mg protein per ml, 0.4–12 μM [^{14}C]ATP, 100 mM KCl, 1 mM EGTA, 10 mM histidine, pH 7.6, and 1.2 mM Mg^{2+} (○—○ and ●—●) or 1 mM CHTA (×—×).

with an inflection point at approx. pH 7.3. K_{MgATP} was calculated (*cf.* legend Fig. 2) assuming that the number of binding sites is independent of pH, rather than determining K_{MgATP} at each pH by a Scatchard plot. Preincubation of the membranes at pH 6 and then carrying out the binding assay at pH 7.5 showed that the lower binding at pH 6.0 is not caused by irreversible inactivation of the enzyme.

The Ca^{2+} pump protein also binds ADP (Table II), however, with a lower affinity than ATP under comparable conditions. This binding is too weak to be quantitatively analyzed using the Millipore filtration technique. It is possible, however, to measure ADP binding indirectly by measuring the displacement of [^{14}C]ATP by ADP in the presence of 1 mM EGTA and 5 mM Mg^{2+}. ADP was somewhat less effective in inhibiting ATP binding when the Mg^{2+} concentration was lowered to 1.2 mM. The inhibition by ADP is compatible with competitive binding of ATP and ADP (Fig. 3). From the intercept and slopes, a K_{MgATP} equal to 0.31 μM^{-1} and apparent ADP inhibition constants of 15 and 19.5 μM (at 35 and 89 μM ADP, respectively) were calculated. The "true" ADP inhibition constant is slightly lower, since in the calculation it was assumed that all of the ADP was unbound and in the form of $MgADP^-$ (ref. 16).

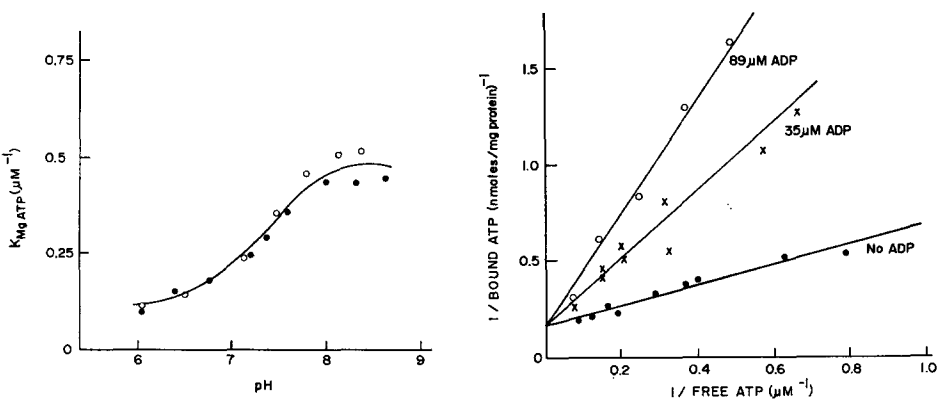


Fig. 2. pH dependence of ATP binding by sarcoplasmic reticulum and Ca^{2+} pump protein. Sarcoplasmic reticulum vesicles (○—○) and Ca^{2+} pump protein (●—●) (9.3 μg bound P per mg), prepared by partial extraction of sarcoplasmic reticulum vesicles with cholate, had phosphoenzyme levels of 6.3 ± 0.3 and 8.1 ± 0.4 nmoles ^{32}P per mg protein, respectively. The binding assay was carried out with 0.25–0.5 mg protein per ml, 1.0–2.5 μM [^{14}C]ATP, 100 mM KCl, 5 mM Mg^{2+}, 1.5 mM EGTA and 10 mM Tris–histidine buffer at the indicated pH. The binding constant, K_{MgATP}, was calculated according to the equation:

$$K_{MgATP} = \frac{N_{bound}}{[ATP]_{free}(N_{total} - N_{bound})}$$

N_{total} = number of binding sites per mg protein as determined by phosphoenzyme assay. N_{bound} = nmoles ATP bound per mg protein. [ATP] in μM.

Fig. 3. The effect of ADP on ATP binding by Ca^{2+} pump protein. Ca^{2+} pump protein, prepared by partial extraction of sarcoplasmic reticulum vesicles with deoxycholate, contained 21 μg bound P per mg protein and had a phosphoenzyme level of 6.0 nmoles ^{32}P per mg protein. The binding assay was carried out with 0.6 mg sarcoplasmic reticulum protein per ml in 100 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 10 mM Tris–histidine buffer (pH 8.0), the indicated concentrations of ATP, and without (●—●), 35 μM (×—×) or 89 μM (○—○) ADP.

TABLE II

ADP BINDING BY Ca^{2+} PUMP PROTEIN

Ca^{2+} pump protein, purified by the cholate procedure and having a phosphoenzyme level of 7 nmoles ^{32}P per mg protein, was incubated in a solution which contained 0.45 mg protein per ml, 1 μM [^{14}C]ADP, 100 mM KCl, 10 mM histidine (pH 7.6) and the indicated concentrations of Mg^{2+}, Ca^{2+}, EGTA and CHTA.

Components added	*ADP binding (nmoles/mg protein)*
0.1 mM Ca^{2+}, 5 mM Mg^{2+}	0.40
1 mM EGTA, 1 mM CHTA	0.45
1 mM EGTA, 1.2 mM Mg^{2+}	0.65
1 mM EGTA, 5 mM Mg^{2+}	0.70

Ca^{2+} binding

Only Ca^{2+} pump protein preparations which did not accumulate Ca^{2+} (less than 0.1 μmole Ca^{2+} per mg protein in the presence of 5 mM oxalate[12]) were used for studying Ca^{2+} binding. We also checked for the presence of a significant pool of non or slowly exchangeable Ca^{2+}. The membranes were incubated for 30 min at 0 °C in the presence of $^{45}Ca^{2+}$ and then centrifuged to obtain a supernatant fraction. The ratio of total to free Ca^{2+} was then determined by atomic absorption spectroscopy and radioactivity measurements of both the complete solution and the supernatant fraction. Both measurements gave, within the error of the experiment, the same ratio, showing that cold Ca^{2+} and $^{45}Ca^{2+}$ had essentially equilibrated. The Millipore filtration technique was not used for routine Ca^{2+} binding studies because exchangeable Ca^{2+} is bound to the Millipore filters (*cf.* Materials and Methods).

Fig. 4 presents two Scatchard plots of Ca^{2+} binding by the Ca^{2+} pump protein at Mg^{2+} concentrations of 1 mM and 5 mM. The data form two straight lines with different slopes. At the higher Mg^{2+} concentration, Ca^{2+} binding affinity is somewhat lower, however, the maximum number of binding sites is about the same in both cases as evidenced by the similar intercept on the abscissa. There is apparently only one type of high-affinity binding site present in the Ca^{2+} pump protein with a binding constant, $K_{Ca}{}^{2+}$, of 0.26 μM^{-1} at 1 mM Mg^{2+} and 0.18 μM^{-1} at 5 mM Mg^{2+}. The intercept on the abscissa gives about 14 nmoles per mg protein as the number of binding sites, or practically twice the number of phosphorylation sites. Thus, in addition to the one binding site for ATP, for each active site in the Ca^{2+} pump protein there appears to be two identical, noninteracting binding sites for Ca^{2+}.

The specificity of the Ca^{2+} binding site for Ca^{2+} was tested by varying the Mg^{2+} concentration and by adding Sr^{2+} or La^{3+} to the medium. As shown in Fig. 5, Mg^{2+}, Sr^{2+} and La^{3+} decrease Ca^{2+} binding in that order. Previously it has been reported that La^{3+} inhibition of Ca^{2+} binding to the Ca^{2+} specific binding sites is of the competitive type[11].

As shown above (Fig. 2), the affinity of the ATP binding site for ATP increases when the pH is raised from 6 to 8. A similar pH dependence is observed for Ca^{2+} binding. Scatchard plots of the data (Fig. 6a) yield two reasonably straight lines at

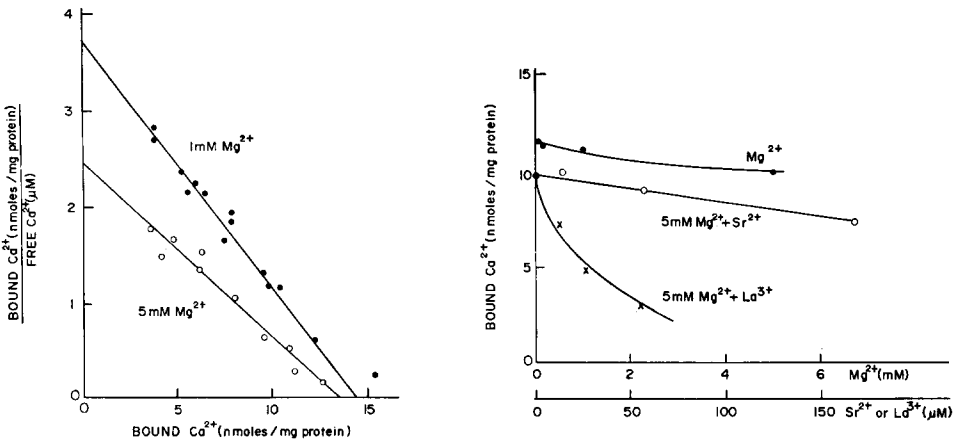


Fig. 4. Scatchard plots of Ca^{2+} binding by Ca^{2+} pump protein. Ca^{2+} pump protein was prepared by partially extracting sarcoplasmic reticulum vesicles with deoxycholate. The preparation contained 21.8 μg bound P per mg protein and had a phosphoenzyme level of 7.0 $\pm$ 0.5 nmoles ^{32}P per mg protein. The Ca^{2+} binding assay was carried out in 2 ml of a solution containing 2 mg sarcoplasmic reticulum protein, 100 mM KCl, 10 mM histidine (pH 7.5), 5–85 μM $^{45}Ca^{2+}$ and 1 mM Mg^{2+} (●—●) or 5 mM Mg^{2+} (○—○).

Fig. 5. Effect of Mg^{2+}, Sr^{2+} and La^{3+} on Ca^{2+} binding by Ca^{2+} pump protein. Ca^{2+} pump protein, prepared by partial extraction of sarcoplasmic reticulum vesicles with cholate, contained 19.8 μg bound P per mg protein and had a phosphoenzyme level of 6.2 nmoles ^{32}P per mg protein. Ca^{2+} binding assay was carried out with 1.1 mg protein per ml, 17 μM $^{45}Ca^{2+}$, 100 mM KCl, 10 mM Tris–histidine buffer (pH 7.4) and the indicated concentrations of Mg^{2+} (●—●), 5 mM Mg^{2+} *plus* Sr^{2+} (○—○) or 5 mM Mg^{2+} *plus* La^{3+} (×—×).

pH 6.35 and 7.4 with practically the same intercept of the abscissa ($n = 13.0$–13.5) and with binding constants of 0.09 μM^{-1} and 0.5 μM^{-1}, respectively. At pH 8.1 the data deviate from linearity, indicating that Ca^{2+} binding becomes more complex at the higher pH. Hill plots of these data give coefficients which decrease from 1.0 at pH 6.35 to 0.85 at pH 7.4 and 0.55 at pH 8.1 (Fig. 6b). Thus a rise in pH from 6.35 to 8.1 appears to have two opposite effects. The binding constant for Ca^{2+} increases while the noninteraction of the two Ca^{2+} binding sites is modified to a negatively cooperative interaction. However, the number of binding sites does not appear to be changed by pH. In Fig. 7 an apparent binding constant, $K'_{Ca}{}^{2+}$, is plotted against pH over the range from 6 to 9 for two different Ca^{2+} pump protein preparations. $K'_{Ca}{}^{2+}$ was estimated from the data of two to three measurements at each pH rather than using Scatchard plots. It was assumed that Ca^{2+} binding is noncooperative and that the number of binding sites is constant over the entire pH range. This is certainly not true at pH 8.1 (Fig. 6b), so that the two graphs in Fig. 7 should be understood to present Ca^{2+} binding by the Ca^{2+} pump protein in a more qualititative rather than quantitative way above pH 7.4. The two preparations of Ca^{2+} pump protein used differed in the amount of phospholipid removed during the detergent treatment of sarcoplasmic reticulum vesicles. The preparation with a lower bound P to protein ratio displays sites with the higher binding affinity. For both preparations a very similar pH profile was obtained. At pH 6 the sites have a low binding affinity for

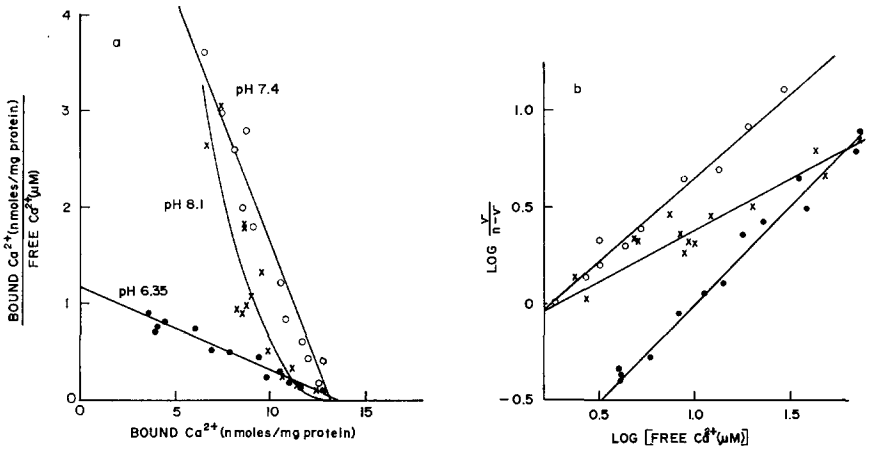


Fig. 6. Scatchard and Hill plots for Ca^{2+} binding by Ca^{2+} pump protein at pH 6.35, 7.4 and 8.1. Ca^{2+} pump protein was prepared by partially extracting sarcoplasmic reticulum vesicles with cholate. The preparation contained 19.8 μg bound P per mg protein and had a phosphoenzyme level of 6.5 ± 0.3 nmoles ^{32}P per mg protein. Ca^{2+} binding assay was carried out in 2 ml of a medium containing 2 mg sarcoplasmic reticulum protein, 100 mM KCl, 5 mM $MgCl_2$, 8–100 μM $^{45}Ca^{2+}$ and 10 mM Tris–histidine buffer at pH 6.35 (●—●), pH 7.4 (○—○) or pH 8.1 (×—×). (a) Scatchard plots, (b) Hill plots. Free Ca^{2+} concentration is given in μM, n (equal to 2) is the number of binding sites per phosphorylation site and

$$v = \frac{\text{Bound } Ca^{2+} \text{ (nmoles/mg protein)}}{\text{Phosphorylation sites (nmoles } ^{32}P\text{/mg protein)}}$$

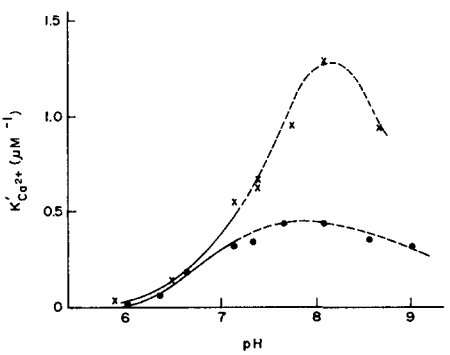


Fig. 7. pH dependence of Ca^{2+} binding by Ca^{2+} pump protein. Ca^{2+} pump protein was prepared by partially extracting sarcoplasmic reticulum vesicles with cholate (1.5 or 1.1 mg cholate per mg protein). The preparations contained 9.3 and 19.8 μg bound P per mg protein, and had phosphoenzyme levels of 8.1 (×—×) and 6.5 (●—●) nmoles ^{32}P per mg protein, respectively. The Ca^{2+} binding assay solution contained 0.85 mg protein per ml, 100 mM KCl, 5 mM Mg^{2+}, 8–14 μM $^{45}Ca^{2+}$ and 10 mM Tris–histidine buffer at the indicated pH. An apparent binding constant, $K'_{Ca^{2+}}$, was calculated according to the equation

$$K'_{Ca^{2+}} = \frac{N_{bound}}{[Ca^{2+}]_{free}(2N_{total} - N_{bound})}$$

N_{total}= number of binding sites per mg protein as determined by phosphoenzyme assay. N_{bound}= nmoles $^{45}Ca^{2+}$ bound per mg protein. $[Ca^{2+}]$ in μM.

Ca^{2+}, while maximal Ca^{2+} binding occurs at pH 8, apparently. The binding constant has half-maximal values at pH 6.7 and 7.3, the higher pH being found for the preparation with the lower amount of residual phospholipid. The changes in binding affinities with pH are reversible within the described pH range. Preincubation at pH 6 or 9 does not change the amount of Ca^{2+} bound at pH 7.5.

Mg^{2+} binding by the Ca^{2+} pump protein

A specific Mg^{2+} binding site with an affinity comparable to those of the Ca^{2+} and ATP binding sites could not be detected for the Ca^{2+} pump protein using a variety of conditions (Table III). Assuming one binding site per phosphorylation site, the binding constant for Mg^{2+} was calculated to be about 0.03 μM^{-1} or less.

TABLE III

Mg^{2+} BINDING BY Ca^{2+} PUMP PROTEIN

Ca^{2+} pump protein was prepared by partially solubilizing sarcoplasmic reticulum vesicles with cholate or deoxycholate. The preparations contained 11.8 and 21.8 μg bound P per mg protein and had phosphoenzyme levels of 8.4 and 7.0 nmoles ^{32}P per mg protein, respectively. Mg^{2+} binding was measured by removing the membranous fraction by centrifugation from a solution which contained 1.1 mg protein per ml, 100 mM KCl, 10 mM Tris–histidine buffer at pH 6.4, 7.3 or 8.1 and the indicated concentrations of Ca^{2+} and Mg^{2+}. Total Mg^{2+} concentration of the suspension and the Mg^{2+} concentration of the supernatant fraction were determined by atomic absorption spectroscopy[12].

Sample	$[Ca^{2+}]_{total}$ *(mM)*	$[Mg^{2+}]_{total}$ *(μM)*	*Mg^{2+} binding (nmoles/mg protein)*		
			pH 6.4	*pH 7.3*	*pH 8.1*
Cholate preparation	0.13	9.8	1.2	1.6	1.0
	0.65	10.2	1.2	1.7	1.5
Deoxycholate preparation	0.13	18.5	0.4	0.5	0.5

Ca^{2+} binding and phosphoenzyme level

Although it has been repeatedly found that phosphoenzyme formation is dependent on Ca^{2+} (refs 13, 17–20), a direct and quantitative connection between Ca^{2+} binding and phosphoenzyme level has not been established. Experiments were performed to obtain such a relation and to determine the effect of phosphoenzyme formation on the binding affinity of the Ca^{2+} binding site. Ca^{2+} binding was determined with $^{45}Ca^{2+}$ in the absence and presence of 1 mM ATP and compared with the level of phosphoenzyme under identical conditions, except that 0.1 mM [^{32}P]ATP instead of 1 mM ATP was used. At the concentrations used, ATP saturates the ATP binding sites. As seen in Table IV, only small changes in the level of bound Ca^{2+} are found upon addition of ATP to the medium. The most noticeable change occurs when only a few sites are occupied by Ca^{2+}, under such conditions there is a small increase in the amount of bound Ca^{2+} in the presence of ATP. The ratio of bound Ca^{2+} (in the presence of ATP) to phosphorylated intermediate is close to two over a 5-fold range of phosphoenzyme concentration. The Ca^{2+} binding experiments in the presence of

TABLE IV

Ca^{2+} BINDING AND PHOSPHOENZYME LEVEL

Ca^{2+} pump protein, prepared by partial extraction of sarcoplasmic reticulum vesicles with cholate, contained 19.4 μg bound P per mg protein and had a maximal phosphoenzyme level of 6.5 nmoles ^{32}P per mg protein. The sample (1 mg sarcoplasmic reticulum protein per ml) was incubated for 30 min at 0 °C in a medium containing 100 mM KCl, 5 mM $MgCl_2$, 10 mM histidine (pH 7.5 or 6.3), and the indicated concentrations of $^{45}Ca^{2+}$ and EGTA to obtain various concentrations of free Ca^{2+}. Aliquots were taken to measure the amount of bound Ca^{2+} both in the presence and absence of 1 mM ATP. The phosphoenzyme level was measured using 0.1 mM [^{32}P]ATP. In the Ca^{2+} binding assay the membranous fraction was removed by centrifugation for 30 min and by Millipore filtration (using washed filters) at 30 s and 1 min after the addition of ATP. In controls up to 20% of the ATP was hydrolyzed within 30 min. All data are the average of at least two determinations.

pH	*Components added*		*Ca^{2+} binding (nmoles/mg protein)*		[^{32}P]*phosphoenzyme (nmoles ^{32}P/mg protein)*
	$[Ca^{2+}]_{total}$ (μM)	[*EGTA*] (μM)	*−ATP*	*+1 mM ATP*	
7.5	17.5	100	0.4	—	0.2
	17.0	27	1.3	—	0.8
	18.0	21	2.4	3.4	—
	18.0	16.5	4.6	6.0	2.9
	18.1	10.5	6.2	6.9	3.4
	16.5	0	10.2	10.3	5.5
	44	0	11.9	11.5	6.1
6.3	17.5	11	5.7	8.3	4.4
	40	0	9.8	11.4	6.3

ATP were not easy to carry out. ATP splitting had to be minimized and it was difficult to evaluate the degree of breakdown of the phosphorylated intermediate in the pellet formed during the centrifugation step which was routinely used to remove the bound Ca^{2+} together with the membranous fraction. In order to minimize ATP hydrolysis, the samples were also processed by the Millipore filtration technique using washed filters. Both procedures gave practically the same results. The data presented in Table IV suggest that phosphoenzyme formation does not appreciably change the affinity of the Ca^{2+} binding sites for Ca^{2+} and that each phosphorylated intermediate contains two Ca^{2+}. In a previous study by Fiehn and Migala[21] it was also found that the total amount of bound Ca^{2+} is approximately the same in the absence and presence of $MgATP^{2-}$.

Determination of the equilibrium constant for phosphoenzyme formation

The ATP–ADP competition experiments shown in Fig. 3 were performed in the presence of EGTA to complex free Ca^{2+}. In studies done in the presence of free Ca^{2+}, a phosphorylated intermediate is rapidly formed upon addition of ATP[13,17,18,20]

$$E_{Ca_2}^{2+} + ATP \overset{Mg^{2+}}{\rightleftharpoons} E\text{–}P_{Ca_2}^{2+} + ADP$$

This reaction is reversible[9,22] and its equilibrium constant can be determined. Since a Ca^{2+} gradient across the membrane would affect the equilibrium[5], these experiments are best carried out with sarcoplasmic reticulum preparations which are unable to accumulate Ca^{2+}, such as the Ca^{2+} pump protein preparation. The assay conditions were chosen to obtain a defined system with a limited number of species involved in the phosphorylation reaction. At a concentration of 0.1 mM, Ca^{2+} essentially saturates the Ca^{2+}-binding sites. At pH 7.5 and higher the phosphate groups of ATP and ADP are practically fully ionized[16,23]. At the Mg^{2+} concentrations (0.6–50 mM) used, 96% or more of the ATP is present as $MgATP^{2-}$ assuming a binding constant of 40000 M^{-1} for the $MgATP^{2-}$ complex[16,23,24]. The binding constant for the $MgADP^{-}$ complex has been reported to range between 1000 M^{-1} and 5000 M^{-1} (refs 16, 24, 25), so that at the Mg^{2+} concentrations used appreciable amounts of free ADP^{3-} and of $MgADP^{-}$ are present.

Fig. 8 presents the data of some of these experiments carried out with Ca^{2+} pump protein at pH 8.0. A plot of the reciprocal of the phosphoenzyme concentration approximates straight lines whose slope and intercept with the ordinate vary with the Mg^{2+} concentration. Similarly a straight line is obtained when these studies were performed at pH 7.5 with Ca^{2+} pump protein. From the intercept with the ordinate and the slope, the maximal level of phosphoenzyme (in the absence of ADP) and an apparent equilibrium constant (K'_{eq}) were calculated (Table V). Extrapolating to zero ADP concentration, the phosphoenzyme concentration is somewhat decreased

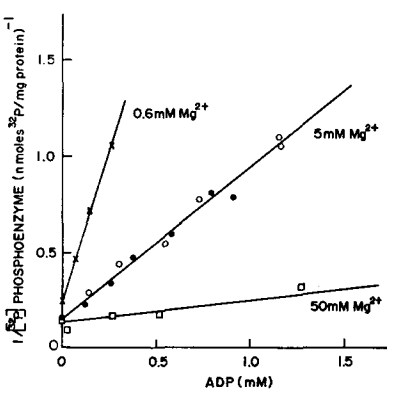


Fig. 8. Determination of the equilibrium constant for phosphoenzyme formation. Ca^{2+} pump protein (19 μg bound P per mg protein), prepared by partial extraction of sarcoplasmic reticulum vesicles with cholate, was incubated at 0 °C in 0.98 ml of a medium which contained 1 mg sarcoplasmic reticulum protein, 100 mM KCl, 0.1 mM $CaCl_2$, 10 mM Tris–histidine, the indicated concentrations of ADP and 0.6 mM (×—×, at pH 8.0), 5 mM (●—●, at pH 8.0, and ○—○, at pH 7.5) or 50 mM (□—□, at pH 8.0) Mg^{2+}. Phosphoenzyme formation was initiated by the addition of 20 μl of 5.65 mM [γ-^{32}P]ATP with rapid stirring and was stopped after 6 s with 25 ml of cold 4.25% trichloroacetic acid containing 0.5 mM ATP and 1 mM P_i and then further processed as described in Methods and Materials. An identical phosphoenzyme level (at pH 7.5) was reached when the reaction was stopped either after 2–3 s or 30 s indicating that the reaction had reached its equilibrium after 6 s. To determine the extent of ATP hydrolysis, charcoal was added to the first supernatant fraction. The amount of radioactivity remaining in solution (approx. 5%) was only 2–3% above that of a control from which sarcoplasmic reticulum protein had been omitted. This indicated that less than 3% of the [γ-^{32}P]ATP was hydrolyzed in the absence and presence of ADP.

at 0.6 mM Mg^{2+} concentration. The apparent equilibrium constant increases with increasing Mg^{2+} concentration. In order to account for the effect of Mg^{2+} on the level of phosphoenzyme, the following two simple reactions were considered:

$$MgATP^{2-} + E_{Ca_2^{2+}} \overset{K_1}{\rightleftharpoons} E\text{–}P_{Ca_2^{2+}} + MgADP^{-} \quad (1)$$

$$MgATP^{2-} + E_{Ca_2^{2+}} \overset{K_{21}}{\rightleftharpoons} E\text{–}P_{Ca_2^{2+},Mg^{2+}} + ADP^{3-} \quad (2)$$

$$E\text{–}P_{Ca_2^{2+},Mg^{2+}} \overset{K_{22}}{\rightleftharpoons} E\text{–}P_{Ca_2^{2+}+Mg^{2+}}$$

The phosphorylated enzyme in Eqn 2 differs from that of Eqn 1 by binding one Mg^{2+} in addition to the two Ca^{2+}. Further, ADP^{3-} and not $MgADP^{-}$ is the substrate of the reverse phosphorylation reaction. Calculation of the equilibrium constants shows that K_{21} and K_{22}, in contrast with K_1 (Table V), are independent of the Mg^{2+} concentrations used in Fig. 8. Substituting the value of 2500 M^{-1} for K_3, the binding constant of the $MgADP^{-}$ complex, K_{21} and K_{22} approximate 0.10 and 1 mM, respectively. Thus the phosphorylation reaction may proceed as described by Eqn 2. It

TABLE V

EQUILIBRIUM CONSTANT OF THE PHOSPHORYLATION REACTION

Maximal levels of phosphoenzyme (E–P_m) and apparent equilibrium constants (K'_{eq}) were calculated according to the equation

$$\frac{1}{E\text{–}P} = \frac{1}{E\text{–}P_m} + \frac{1}{E\text{–}P_m} \cdot \frac{1}{K'_{eq}} \cdot \frac{[ADP]_{total}}{[ATP]_{total}}$$

Ca^{2+} pump protein and sarcoplasmic reticulum vesicles were phosphorylated as described in the legend of Fig. 8. "True" equilibrium constants (K_1, K_{21} and K_{22}) were estimated according to the equations

$$K_1 = \frac{K_3[Mg] \cdot K'_{eq}}{1 + K_3[Mg]}\,; \quad K_{21} = \frac{K'_{eq}}{(K_3[Mg] + 1)\left(1 + \frac{K_{22}}{[Mg]}\right)}$$

where $K_3 = ([MgADP]/[Mg][ADP])$. The value of K_3 was taken to be 2500 M^{-1} (refs 16, 24, 25).

Preparation	*pH*	*Mg^{2+} (mM)*	*[^{32}P]Phosphoenzyme (nmoles ^{32}P/mg protein) ([ADP]= 0)*	*K'_{eq}*	*K_1*	*K_{21}*	*K_{22} (mM)*
Ca^{2+} pump protein	8.0	0.6	4.8	0.56	0.34	0.08	1.0
	8.0	5.0	6.7	1.7	1.6	0.10	1.0
	8.0	50.0	6.9	12.1	12.0	0.09	1.0
	7.5	5.0	6.6	1.7	1.6	0.10	1.0
Sarcoplasmic reticulum vesicles	7.5	5.0	6.4	1.6	1.5	0.10	1.0

may be noted that in the calculation it was assumed that all of the Mg^{2+} is present in the uncomplexed form. This assumption does not significantly change the values for K_{21} and K_{22} as long as the Mg^{2+} concentration is high relative to the ADP concentration, which is the case in most of the experiments shown in Fig. 8. The two equilibrium constants are not appreciably changed when the ATP concentration is changed from 0.113 mM (Fig. 8) to either 0.05 or 0.5 mM (not shown). These experiments were carried out in the presence of 5 mM Mg^{2+} at pH 7.5. Table V also contains data obtained at pH 7.5 with sarcoplasmic reticulum vesicles using 5 mM Mg^{2+}. Essentially the same results were found indicating that the detergent treatment used to prepare the Ca^{2+} pump protein does not appreciably alter the equilibrium of the phosphorylation reaction.

Effect of N-ethylmaleimide

In previous studies it has been found that *N*-ethylmaleimide inhibits Ca^{2+}-stimulated ATPase activity and Ca^{2+} uptake indicating that sulfhydryl groups are involved in Ca^{2+} pump activity[18,26,27]. To test which of the several possible steps in ATPase activity are blocked, the effect of *N*-ethylmaleimide on Ca^{2+} and ATP binding and phosphoenzyme formation was investigated (Table VI). *N*-ethylmaleimide

TABLE VI

THE EFFECT OF *N*-ETHYLMALEIMIDE ON Ca^{2+} BINDING, ATP BINDING AND PHOSPHOENZYME FORMATION BY Ca^{2+} PUMP PROTEIN

Ca^{2+} pump protein, prepared by partial extraction of sarcoplasmic reticulum vesicles with deoxycholate (Sample A) or cholate (Sample B), was incubated at 23 °C for 10 min in a medium containing 1.4 mg sarcoplasmic reticulum protein/ml, 1 mM *N*-ethylmaleimide, 100 mM KCl, 10 (Sample A) or 20 (Sample B) μM Ca^{2+}, and 10 mM Tris–histidine buffer (pH 8.5). *N*-Ethylmaleimide was omitted from control samples. To measure Ca^{2+} and ATP binding and phosphoenzyme formation, aliquots were then taken and diluted with a Tris–histidine buffer (pH 5.1) containing the appropriate amounts of KCl, $MgCl_2$, $CaCl_2$ and EGTA. Ca^{2+} binding was carried out with a solution which contained 1 mg protein per ml, 11.2 (Sample A) or 18.5 (Sample B) μM $^{45}Ca^{2+}$, 100 mM KCl, 5 mM Mg^{2+}, and 10 mM Tris–histidine buffer, pH 8.0. ATP binding was measured using a solution which contained 0.65 mg sarcoplasmic reticulum protein per ml, 3 μM [^{14}C]ATP, 100 mM KCl, 5 mM Mg^{2+}, 6 μM Ca^{2+} (Sample A) or 10 μM Ca^{2+} (Sample B), 2 mM EGTA and 10 mM Tris–histidine, pH 7.9. To determine the phosphoenzyme level a third aliquot was diluted to give a solution with 1 mg sarcoplasmic reticulum protein per ml containing 100 mM KCl, 0.1 mM Ca^{2+}, 5 mM Mg^{2+} and 10 mM Tris–histidine buffer, pH 8.0. The same levels of phosphoenzyme were found in control samples to which *N*-ethylmaleimide was added directly before the addition of [^{32}P]ATP.

Sample	*Ca^{2+} binding (nmoles/mg protein)*	*ATP binding (nmoles/mg protein)*	*[^{32}P]Phosphoenzyme (nmoles ^{32}P/mg protein)*
Deoxycholate preparation			
−*N*-ethylmaleimide	5.0	2.2	5.5
+*N*-ethylmaleimide	2.4	0.25	0.15
Cholate preparation			
−*N*-ethylmaleimide	13.2	2.6	8.0
+*N*-ethylmaleimide	9.4	0.1	0.20

clearly inhibits ATP binding, and phosphoenzyme formation is consequently inhibited. Ca^{2+} binding is decreased to a lesser degree. One of the samples treated with *N*-ethylmaleimide still bound 9.4 nmoles of Ca^{2+} per mg protein indicating that the binding affinity and not the number of binding sites is affected. To establish this with more certainty, however, a complete binding curve would have to be obtained.

pH dependence of phosphoenzyme formation and ATPase activity

Figs 2 and 7 illustrate that Ca^{2+} and ATP binding are greatly influenced by the pH of the medium. These studies were extended by determining the pH profiles of phosphoenzyme formation and ATPase activity (Fig. 9). To eliminate the pH effect on Ca^{2+} and ATP binding in these experiments, conditions were used which resulted in essentially complete saturation of both sites with their respective substrate above pH 6.5. Under these conditions, phosphoenzyme formation is largely independent of the pH over the range tested. ATPase activity on the other hand displays a distinct dependence on pH with a maximum at about pH 7.3. Because phosphoenzyme formation is independent of pH the curve in Fig. 9 likely represents the pH profile of one or more of the subsequent steps in Ca^{2+} pump activity which lead to hydrolysis of the phosphorylated intermediate to yield enzyme *plus* P_i.

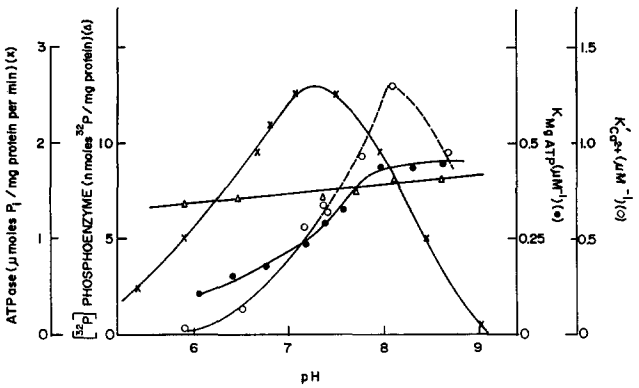


Fig. 9. pH dependence of Ca^{2+} and ATP binding, phosphoenzyme formation and ATPase activity of Ca^{2+} pump protein. The Ca^{2+} pump protein was prepared by partially extracting sarcoplasmic reticulum vesicles with cholate. The preparation contained 9.3 μg bound P per mg protein and had a phosphoenzyme level of 8.1 nmoles ^{32}P per mg protein. The Ca^{2+} binding (○) and ATP binding (●) assays were carried out as described in Figs 2 and 7, respectively. Phosphoenzyme level (△) and Ca^{2+} stimulated ATPase activity (×) were measured as described in Methods and Materials except that a 10 mM Tris–histidine buffer of varying pH was used.

Effect of Mg^{2+}, Sr^{2+}, and La^{3+} on the level of phosphoenzyme and ATPase activity

In Fig. 5 we showed that Mg^{2+}, Sr^{2+} and La^{3+} decrease, in that order, Ca^{2+} binding by the Ca^{2+} pump protein. Some preliminary experiments were carried out to test whether the addition of these cations also affects the level of phosphoenzyme and ATPase activity (Table VII). Addition of Sr^{2+} to the medium does not appreciably change the level of phosphoenzyme, although the amount of bound Ca^{2+} decreases more than two-fold (in the absence of ATP). ATPase activity is slightly decreased in the presence of 0.75 mM Sr^{2+} at the low level of Ca^{2+} present in the assay medium. These findings agree with earlier studies which have shown that Sr^{2+} may support ATPase activity and can be transported by sarcoplasmic reticulum[3]. Addition of

La^{3+}, which also lowers the amount of Ca^{2+} bound, decreases, on the other hand, the level of phosphoenzyme and ATPase activity appreciably. The decrease in both activities, is not proportional to the decrease in Ca^{2+} binding, possibly because phosphoenzyme formation may result in an increase of the specificity of the Ca^{2+} binding sites for Ca^{2+}. In agreement with the data presented in Table V, a decrease in Mg^{2+} concentration from 5 mM to approx. 0.02 mM results in a lower level of phosphoenzyme. Ca^{2+} binding is not affected by the change in Mg^{2+} concentration.

TABLE VII

THE EFFECT OF Mg^{2+}, Sr^{2+} AND La^{3+} ON PHOSPHOENZYME FORMATION AND ATPase ACTIVITY

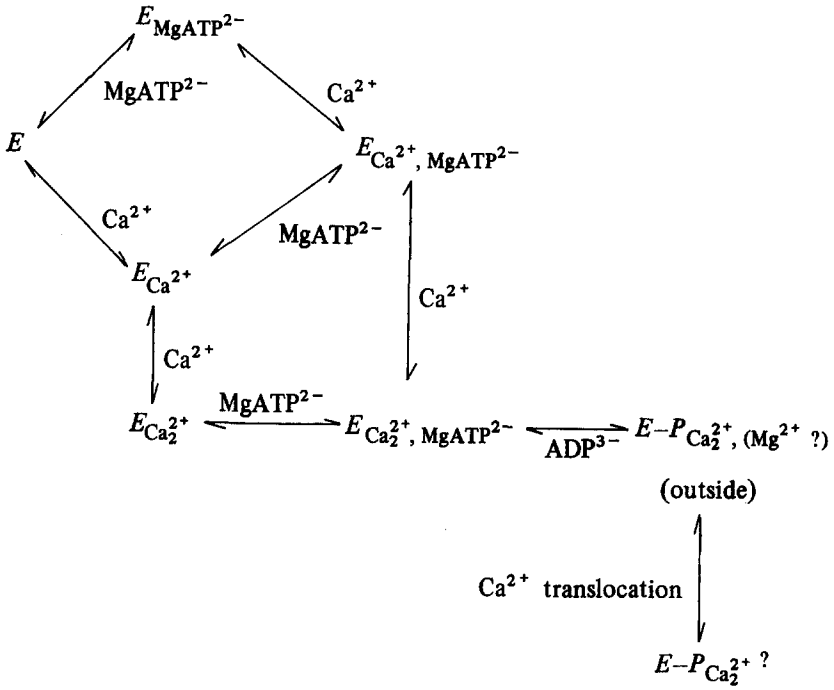
Ca^{2+} pump protein, prepared by partial extraction of sarcoplasmic reticulum vesicles with cholate, contained 19 μg bound P per mg protein and had a phosphoenzyme level of 7.0 nmoles ^{32}P per mg protein. Samples were incubated at 0 °C for 30 min in a medium containing 1 mg sarcoplasmic reticulum protein per ml, 100 mM KCl, 10 mM histidine (pH 7.6) and the indicated concentrations of Mg^{2+}, Ca^{2+}, Sr^{2+} and La^{3+}. Aliquots were taken to measure Ca^{2+} binding (in the absence of ATP) and the level of phosphoenzyme. ATPase activity was estimated as described in Methods and Materials except that the medium contained the indicated concentrations of Mg^{2+}, Ca^{2+}, Sr^{2+} and La^{3+}.

Components added				*Ca^{2+} binding (nmoles/mg protein)*	*[^{32}P]Phosphoenzyme (nmoles ^{32}P/mg protein)*	*ATPase activity (μmoles P_i/mg protein per min)*
Mg^{2+} (mM)	*Ca^{2+} (μM)*	*Sr^{2+} (mM)*	*La^{3+} (mM)*			
5	100			—	6.5	4.8
5	13			10	4.8	5.0
5	13	0.8		4	5.6	4.4
5	12		0.08	1.0	2.8	3.7
5	12		0.5	0.6	0.7	0.5
≈0.02	17			10.8	2.5	—

DISCUSSION

The binding of ATP and Ca^{2+} to the Ca^{2+} pump protein of sarcoplasmic reticulum is reported and compared to phosphoenzyme formation. These studies were carried out using preparations of partially purified Ca^{2+} pump protein rather than native sarcoplasmic reticulum vesicles for several reasons. While ATP binding could be effectively studied with either preparation, use of the native sarcoplasmic reticulum vesicles would have made the Ca^{2+} binding studies extremely difficult, since they contain an appreciable number of additional nonspecific high-affinity Ca^{2+} binding sites and a significant pool of not rapidly exchangeable Ca^{2+} (refs 11, 12, 28). Also, it would have been difficult to distinguish between Ca^{2+} uptake and Ca^{2+} binding of sarcoplasmic reticulum vesicles in experiments carried out in the presence of ATP.

To facilitate the further discussion, the sequence of events in Ca^{2+} transport by sarcoplasmic reticulum, as they follow from the present study, is presented in Scheme 1.



Scheme 1.

The Ca^{2+} pump protein and its phosphorylated intermediate are symbolized by E and E–P, respectively. $E_{Ca}{}^{2+}$, $E_{MgATP}{}^{2-}$, $E_{Ca}{}^{2+}$, $_{MgATP}{}^{2-}$, *etc.* represent Ca^{2+} pump protein molecules with one or more substrate molecules bound to it as indicated by the subscript.

The ATP binding site is present at the phosphorylation site, since (1) the quantity of ATP binding sites is equal to the number of phosphorylation sites, (2) phosphoenzyme formation blocks ATP binding, (3) phosphoenzyme formation and ATP binding are both optimal in the presence of Mg^{2+}, (4) inhibition of ATP binding results in loss of phosphoenzyme formation, and (5) ATP binding is rather specific, as indicated by an approximate 5-fold weaker binding affinity for ADP by sarcoplasmic reticulum membranes. The binding of the two Ca^{2+} is of physiological importance for phosphoenzyme formation since (1) the quantity of Ca^{2+} binding sites is twice the number of phosphorylation sites, (2) there is a close correlation between Ca^{2+} binding and phosphoenzyme formation, (3) the binding site is rather specific with respect to other cations such as Mg^{2+} and Sr^{2+}, and (4) replacement of Ca^{2+} by La^{3+} decreases phosphoenzyme formation as well as ATPase activity.

Since ATP and Ca^{2+} binding and formation of the phosphoenzyme are rapid relative to the hydrolysis of the phosphoenzyme[7,8,20,29], the equilibrium constant, K_{eq}, of the phosphorylation reaction can be estimated. In a previous study Panet *et al.*[7] reported K_{eq} to be close to one. In their calculation of K_{eq}, they assumed that all of the ADP was free, although their experiments were carried out in the presence of approx. 1 mM Ca^{2+} which would complex part of the ADP. Also, the effect of Mg^{2+} on the level of phosphoenzyme was not studied by these authors.

The K_{eq} reported here is for the phosphorylation of the $E_{Ca_2}{}^{2+}$ complex since Ca^{2+} was present at saturating concentrations. Providing that the mechanism is as described by Eqn 2, K_{eq} is less than one (Table V). Thus $\Delta G°$ of formaton for phosphoenzyme is more positive than that for ATP by approx. 1 kcal/mole. It appears then that the translocation step occurs after the phosphoenzyme is formed, implying that the binding and phosphorylation reactions described take place on the outside of the membrane. Further evidence for the formation of phosphoenzyme prior to Ca^{2+} translocation has been reported by Balzer *et al.*[30], who found that certain inhibitors, such as prenylamine, block Ca^{2+} uptake and ATPase activity but not phosphoenzyme formation. A subsequent step, possibly the translocation of the two Ca^{2+} from the outside to the inside of the membrane, would then consume most of the free energy. Since the translocation step likely involves conformational changes of the membrane, it is reasonable to expect that this step is a rate-limiting step of the overall reaction.

The reaction scheme shown above presents several pathways for the formation of an $E_{Ca_2}{}^{2+}, {}_{MgATP}{}^{2-}$ complex which appears to be a direct precursor of the phosphorylated intermediate. Both $MgATP^{2-}$ and Ca^{2+} bind to Ca^{2+} pump protein in the absence of the other substrate, indicating that binding occurs randomly. Based on kinetic studies, Kanazawa *et al.*[8] similarly concluded that binding of $MgATP^{2-}$ and Ca^{2+} follows a random sequence. Further their kinetic studies furnish the best published evidence for the requirement of two Ca^{2+} for phosphoenzyme formation. They found that there is a linear relationship between the reciprocals of the square of the free Ca^{2+} concentration and the maximum initial rate of phosphoenzyme formation. The present study supports this conclusion since as shown in Table IV, once the phosphorylated intermediate is formed, two Ca^{2+} are bound to the active site. This is also consistent with previous studies which show that one ATP molecule is consumed or formed during the translocation of two Ca^{2+} (refs 3, 5).

The role of Mg^{2+} in Ca^{2+} transport by sarcoplasmic reticulum is rather complex and is not yet well understood. With some exceptions[7,19], it has been found that Mg^{2+} is required for the phosphorylation reaction[8,17,18]. It is also believed to have a role in activating the breakdown of the phosphorylated intermediate[7,8,20]. The present study indicates that phosphorylation of sarcoplasmic reticulum proceeds *via* an $E_{Ca}{}^{2+}, {}_{MgATP}{}^{2-}$ and $E_{Ca_2}{}^{2+}, {}_{MgATP}{}^{2-}$ complex. Mg^{2+} is required for maximal ATP binding (Fig. 1). It therefore appears unlikely that ATP^{4-} bound to the membrane in the absence of Mg^{2+} (Fig. 1 and Table I) is a true substrate for the phosphorylation reaction, as it seems to be for the (Na^+,K^+)-ATPase[31]. The steps following the formation of the $E_{Ca_2}{}^{2+}, {}_{MgATP}{}^{2-}$ complex are also dependent on Mg^{2+} (Table VII), however, in a more complex way (Table V) than previously assumed[1,2,8]. Our results indicate that a phosphoenzyme is formed which contains not only two Ca^{2+} binding sites but also an additional Mg^{2+} binding site. The latter might be present in the unphosphorylated Ca^{2+} pump protein as well (Table III).

The affinity of the specific ATP and Ca^{2+}-binding sites for their respective substrates is approximately the same for both sarcoplasmic reticulum vesicles and the Ca^{2+} pump protein. Therefore the ATP and Ca^{2+} binding in sarcoplasmic reticulum is referable to the Ca^{2+} pump protein. Further it would appear that the detergent treatment used in the isolation of the Ca^{2+} pump protein does not appreciably change its binding characteristics. In Table VIII we compare the binding of ATP and Ca^{2+} to the Ca^{2+} pump protein with the data of binding and kinetic studies

TABLE VIII

COMPARISON OF DISSOCIATION AND MICHAELIS CONSTANTS FOR Ca^{2+} AND $MgATP^{2-}$ BY Ca^{2+} PUMP PROTEIN AND SARCOPLASMIC RETICULUM VESICLES

Substrate	*K* (μM)	*pH*	*Remarks***	*Ref.*
Ca^{2+}	1.3	6.5	K_D of sarcoplasmic reticulum–Ca^{2+} complex	11
	0.67–2.0	7.4	determined by equilibrium dialysis in presence of $MgCl_2$ and KCl and absence of ATP	12
	2.5–5.0	7.0	K_D of Ca^{2+} pump protein–Ca^{2+} complex	
	1.4–2.5	7.4	in absence of ATP	Present work
	3	7.2	K_m of Ca^{2+} transport by human sarcoplasmic reticulum vesicles	6
	12	7.2	K_m of Ca^{2+} transport by rat sarcoplasmic reticulum vesicles	6
	4.25*	7.0	K_D of enzyme–Ca^{2+} complex, determined from initial rates of phosphoenzyme formation	8
ATP	4.0–5.0	7.0	K_D of Ca^{2+} pump protein–$MgATP^{2-}$ complex in absence of free Ca^{2+}	Present work
	4.6	7.0	K_m of phosphoenzyme formation, determined from initial rates of phosphoenzyme formation	8
	30	7.0	at 0.05 μM Ca^{2+}	
	4	7.0	at 20 μM Ca^{2+}	K_m of ATPase of sarcoplasmic reticulum 7
ATP	2.0	8.5	K_D of Ca^{2+} pump protein–$MgATP^{2-}$ complex in absence of free Ca^{2+}	Present work
	0.96	8.8	K_m of phosphoenzyme formation, determined from initial rates of phosphoenzyme formation	8
	170	8.5	at $Ca^{2+} < 10^{-8}$ M	K_D of ATPase protein–ATP complex, determined by alkylating ATPase protein with *N*-ethylmaleimide 7
	20	8.5	at 10 μM Ca^{2+}	

* $K_{Ca^{2+}-EGTA}$ has been assumed to be 0.5 μM.
** K_m, Michaelis constant. K_D, dissociation constant.

carried out with sarcoplasmic reticulum vesicles by others. With the exception of the binding data of Panet *et al.*[7], quite remarkable agreement prevails between the dissociation and Michaelis constants. It seems then that the binding constants for Ca^{2+} and $MgATP^{2-}$ are not significantly altered by the binding of the other substrate to the active site.

The variations in the binding constants for Ca^{2+} and ATP with pH suggest that binding involves a group at the active site with a pK of approx. 7.3. The pH profile of ATP binding shown in Fig. 2 is most likely little affected by the ionization of ATP, since in the presence of Mg^{2+} a pK of about 5.3 has been reported for the ionization of $ATPHMg^{-}$ to $ATPMg^{2-} + H^{+}$ (ref. 16). Of the amino acid side chains, histidine would appear to be the most likely possibility, especially since photo-oxidation with Rose Bengal inhibits ATPase activity and Ca^{2+} uptake in sarcoplasmic reticulum[32]. Cysteine has also been shown to be at the active site of Ca^{2+} stimulated ATPase activity by using *N*-ethylmaleimide as inhibitor[18,26,27]. This is consistent with the pH profile of ATPase activity which is dependent on a group with a pK of approx. 8.2 (Fig. 9 and ref. 33). *N*-Ethylmaleimide also inhibits the binding of ATP and formation of phosphoenzyme (Table VI). At present it is not clear whether this results from steric hindrance of ATP binding by *N*-ethylmaleimide or from the involvement of cysteine in these reactions.

It may be significant that the pH of muscle cells[34,35] and the pK values for ATP and Ca^{2+} binding are close to 7. Streter[36] reported that in the presence of ATP, total Ca^{2+} binding by sarcoplasmic reticulum vesicles is also dependent on pH and that it is optimal at about pH 6 in the absence of oxalate. pH changes which have been reported to occur during the excitation event[37] could conceivably affect Ca^{2+} binding and Ca^{2+} transport processes by sarcoplasmic reticulum. In this regard Nakamaru and Schwartz[38] found that an increase in pH from 6.46 to 7.82 results in Ca^{2+} release of preloaded vesicles, while a decrease in pH enhances Ca^{2+} uptake by sarcoplasmic reticulum. The amount of bound ATP, measured in the absence of a Ca^{2+} chelating reagent, was not altered by a change in pH. Our studies show on the other hand an increase in ATP and Ca^{2+} binding affinities with increasing pH. Consequently a possible increase in pH during the excitation event would affect sarcoplasmic reticulum in two ways. At the higher pH, Ca^{2+} could be readily released to initiate muscle contraction. At the same time an increase in ATP and Ca^{2+} binding affinities would occur which would allow a rapid reabsorption of the released Ca^{2+} by the pumping machinery of sarcoplasmic reticulum. Participation of H^{+} in the regulation of Ca^{2+} transport by sarcoplasmic reticulum is an attractive hypothesis. Verification would require the demonstration of selective and rapid pH changes within the muscle cell.

ACKNOWLEDGEMENTS

I am grateful to Dr Sidney Fleischer for his encouragement and advice during the course of this work. I would like to thank Dr Warren L. Zahler for his help in the preparation of the manuscript. Also, I wish to thank Mrs Ikuko Ishii for her excellent technical assistance. These studies were supported by a Special Fellowship of the U.S. Public Health Service (CA 41263), a U.S. Public Health Service Grant AM-14632 and Grants-in-Aid from the Tennessee Heart and Middle Tennessee Heart Associations. G.M. is an established investigator of the American Heart Association.

REFERENCES

1 Martonosi, A. (1971) in *Biomembranes* (Manson, L. A., ed.), Vol. 1, pp. 191–256, Plenum Press, New York

2 Inesi, G. (1972) *Annu. Rev. Biophys. Bioeng.* Vol. 1, pp. 191–210
3 Weber, A., Herz, R. and Reiss, I. (1966) *Biochem. Z.* 345, 329–369
4 Hasselbach, W. and Makinose, M. (1961) *Biochem. Z.* 333, 518–528
5 Makinose, M. and Hasselbach, W. (1971) *FEBS Lett.* 12, 271–272
6 Worsfold, M. and Peter, J. B. (1970) *J. Biol. Chem.* 245, 5545–5552
7 Panet, R., Pick, U. and Selinger, Z. (1971) *J. Biol. Chem.* 246, 7349–7356
8 Kanazawa, T., Yamada, S., Yamamoto, T. and Tonomura, Y. (1971) *J. Biochem. Tokyo* 70, 95–123
9 Inesi, G. and Almendares, J. (1968) *Arch. Biochem. Biophys.* 126, 733–735
10 Ebashi, S. and Lipmann, F. (1962) *J. Cell Biol.* 14, 389–400
11 Chevallier, J. and Butow, R. A. (1971) *Biochemistry* 10, 2733–2737
12 Meissner, G., Conner, G. E. and Fleischer, S. (1973) *Biochim. Biophys. Acta* 298, 246–269
13 Meissner, G. and Fleischer, S. (1971) *Biochim. Biophys. Acta* 241, 356–378
14 Crane, R. K. and Lipmann, F. (1953) *J. Biol. Chem.* 201, 235–243
15 Post, R. L. and Sen, A. K. (1967) in *Methods in Enzymology* (Estabrook, R. W. and Pullman, M. E., eds), Vol. 10, pp. 773–776, Academic Press, New York
16 Phillips, R. C., George, P. and Rutman, R. J. (1966) *J. Am. Chem. Soc.* 88, 2631–2640
17 Yamamoto, T. and Tonomura, Y. (1968) *J. Biochem. Tokyo* 64, 137–145
18 Makinose, M. (1969) *Eur. J. Biochem.* 10, 74–82
19 Martonosi, A. (1969) *J. Biol. Chem.* 244, 613–620
20 Inesi, G., Maring, E., Murphy, A. J. and MacFarland, B. H. (1970) *Arch. Biochem. Biophys.* 138, 285–294
21 Fiehn, W. and Migala, A. (1971) *Eur. J. Biochem.* 20, 245–248
22 Makinose, M. (1966) *Biochem. Z.* 345, 80–86
23 Heyde, M. E. and Rimai, L. (1971) *Biochemistry* 10, 1121–1128
24 Belaich, J. P. and Sari, J. C. (1969) *Proc. Natl. Acad. Sci. U.S.* 64, 763–770
25 Collier, H. B. and Lam, A. (1970) *Biochim. Biophys. Acta* 222, 299–306
26 Hasselbach, W. and Seraydarian, K. (1966) *Biochem. Z.* 345, 159–172
27 Panet, R. and Selinger, Z. (1970) *Eur. J. Biochem.* 14, 440–444
28 Barlogie, B., Hasselbach, W. and Makinose, M. (1971) *FEBS Lett.* 12, 267–268
29 Meissner, G. and Fleischer, S. (1972) *Biochim. Biophys. Acta* 255, 19–33
30 Balzer, H., Makinose, M. and Hasselbach, W. (1968) *Naunyn-Schmiedebergs Arch. Pharmakol. Exp. Pathol.* 260, 444–455
31 Hegyvary, C. and Post, R. L. (1971) *J. Biol. Chem.* 246, 5234–5240
32 Yu, B. P. and Masoro, E. J. (1971) *Fed. Proc.* 30, 1170 Abs.
33 MacLennan, D. H. (1970) *J. Biol. Chem.* 245, 4508–4518
34 Saborowski, F., Usinger, W. and Albers, C. (1971) *Pluegers Arch. Eur. J. Physiol.* 328, 121–134
35 Waddell, W. J. and Bates, R. G. (1969) *Physiol. Rev.* 49, 285–329
36 Streter, F. A. (1969) *Arch. Biochem. Biophys.* 134, 25–33
37 Stephens, W. G. S. (1969) *Nature* 224, 547–549
38 Nakamaru, Y. and Schwartz, A. (1972) *J. Gen. Physiol.* 59, 22–32