Modulation of Calcium Binding in Sarcoplasmic Reticulum Adenosinetriphosphatase†

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ABSTRACT: High-affinity calcium binding to sarcoplasmic reticulum (SR) ATPase occurs with a stoichiometric ratio of 2 with respect to sites phosphorylated with ATP [Yamamoto, T., & Tonomura, Y. (1967) *J. Biochem. (Tokyo) 62*, 558; Yamamoto, T., & Tonomura, Y. (1968) *J. Biochem. (Tokyo) 64*, 137; Makinose, M. (1969) *Eur. J. Biochem. 10*, 74] in steady-state conditions, but with a stoichiometric ratio of 1 with respect to the total number of sites available for phosphorylation with P_i in the absence of Ca^{2+} [Masuda, H., & de Meis, L. (1973) *Biochemistry 12*, 4581] in equilibrium conditions. Additional cation binding sites of intermediate and low affinity for Ca^{2+} are also present in the enzyme. The cooperative character of calcium binding noted with SR vesicles [Inesi, G., Kurzmack, M., Coan, C., & Lewis, D. (1980) *J. Biol. Chem. 255*, 3025] is observed with purified ATPase as well. A cooperative behavior is also displayed by the Ca^{2+} concentration dependence of ATPase enzymatic activity. This cooperativity is abolished, and the apparent affinity for Ca^{2+} is reduced by a detergent known to dissociate ATPase into enzymatically active single chains [Dean, W., & Tanford, C. (1978) *Biochemistry 17*, 1683]. Stoichiometry and cooperative behavior demonstrate that within one enzyme unit two interacting sites are occupied by calcium, while only one out of two phosphorylation sites is phosphorylated by ATP in steady-state conditions. The effect of detergent solubilization indicates that single polypeptide chains retain enzymatic activity which is dependent on the occupancy of noninteracting calcium site(s). Therefore, the cooperative behavior observed with native enzyme is due to chain–chain interactions, or reversible segmental interactions within one chain. The affinity of calcium binding to ATPase is increased by a rise in pH but is not significantly affected by changes in temperature or by ATP analogues which do not phosphorylate the enzyme. Analysis of the pH effect indicates that one group, dissociating with $pK^{app} = 7.3$, participates as a ligand for calcium site modulation during the transport cycle. Ca^{2+} exchange with H^+ is an integral part of the active transport mechanism [Chiesi, M., & Inesi, G. (1980) *Biochemistry 19*, 2912]. Enzyme phosphorylation with ATP in native vesicles is followed by transfer of bound calcium into a location that is not accessible to the medium [Inesi, G., Kurzmack, M., & Verjovski-Almeida, S. (1978) *Ann. N.Y. Acad. Sci. 307*, 224]. On the other hand, when leaky vesicles are used, a stoichiometric release of two calcium ions per enzyme unit is observed following phosphorylation with ATP, consistent with reduction of site affinity for the divalent cation [Ikemoto, N. (1975) *J. Biol. Chem. 250*, 7219]. Observation of the release phenomenon requires addition of dimethyl sulfoxide to the medium, in order to inhibit rapid hydrolytic cleavage of the phosphoenzyme following a slow calcium release [Takakuwa, Y., & Kanazawa, T. (1979) *Biochem. Biophys. Res. Commun. 88*, 1209; Dupont, Y. (1980) *Eur. J. Biochem. 109*, 231]. Therefore, changes in site orientation and reduction in affinity are identified with the mechanistic step utilizing free energy for vectorial transport of Ca^{2+} against a concentration gradient. In the normal operation of the pump, conversion of the phosphoenzyme and inward calcium release from the nonaccessible ("occluded") sites is a slow step which is rapidly followed by hydrolytic cleavage of the phosphoenzyme.

High-affinity calcium binding to sarcoplasmic reticulum (SR) ATPase constitutes an obligatory step for activation of the enzyme and initiation of the catalytic and transport cycle (Tada et al., 1978; de Meis & Vianna, 1979; Inesi, 1979). It was recently reported that this calcium binding exhibits a cooperative behavior and is accompanied by a protein conformational change (Inesi et al., 1980). We describe here experiments designed to clarify the stoichiometry of binding sites with respect to ATPase chains and phosphorylation sites, and the modulation of calcium binding by pH, temperature, simple nucleotide binding, and ATP utilization for active transport.

Materials and Methods

SR vesicles were obtained from rabbit hind leg white muscle as previously described (Eletr & Inesi, 1972). Purified ATPase was prepared by the method of MacLennan (1970) and passed through a Bio-Gel P10 column for removal of residual deoxycholate. Only preparations sustaining hydrolysis of 12–20 μmol mg^{-1} min^{-1} (pH 7.5, 37 °C) were used in our experiments. Alternatively, ATPase was purified according to Meissner et al. (1973).

Calcium binding was measured by the following methods: (1) equilibrium in chromatography columns as previously described (Inesi et al., 1980); (2) equilibrium in dialysis bags containing approximately 2.5 mg of protein in 0.5-mL volume, shaken for 4 h in 50 mL of medium composed of 20 mM 3-(*N*-morpholino)propanesulfonic acid (Mops) (pH 6.9) or 2-(*N*-morpholino)ethanesulfonic acid (Mes) (pH 6.1), 80 mM KCl, 5 mM $MgCl_2$, 45 μM $^{45}CaCl_2$, and different concentrations of ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA); (3) filtration in transient and steady-state conditions with a technique similar to that of Yamaguchi & Tonomura (1979). Volumes (1.2 mL) of reaction mixture containing 20 mM Mes (pH 6.1), 80 mM KCl, 5 mM $MgCl_2$, 50 μM $^{45}CaCl_2$, 10 mM [^{3}H]glucose, and 0.5 mg of ATPase were incubated at 25 °C for 2 min, and two 0.1-mL samples were placed on a Millipore filter (13-mm diameter, 0.45-μm pore size) prewashed with nonradioactive medium. More samples were taken at serial time intervals following addition of ATP or other substrates. The filters were

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then counted to determine the amount of total calcium (^{45}Ca tracer) and the volume of medium ([^{3}H]glucose) retained by the filters. No significant interaction of ^{45}Ca or [^{3}H]glucose with the filter was observed, and no protein leak through the filter was detected. ^{45}Ca and ^{3}H were counted separately by differential settings on a scintillation spectrometer, and the amount of calcium bound to the protein was calculated by subtracting the free calcium in the medium trapped in the filter from the total calcium retained by the filter. For these calculations, the concentration of free calcium in the trapped volume was assumed to be equal to that of the medium. In separate experiments, it was found that this assumption introduces a maximal error of 10%. The filtration time was 5 s.

Spectrophotometric measurements of Ca^{2+} transients were obtained on an Aminco double-wavelength spectrophotometer (585 vs. 575 nm), using Arsenazo III as a metallochromic indicator. The reaction mixture contained 20 mM Mes (pH 6.1), 80 mM KCl, 5 mM $MgCl_2$, 50 μM $CaCl_2$, 50 μM Arsenazo III (pretreated with Chelex), and 0.5 mg of protein/mL. The reaction was started by the addition of ATP.

ATPase activity was followed by determining P_i with the molybdovanadate reaction (LeCocq & Inesi, 1966) as described by Lin & Morales (1977).

ATPase phosphorylation with [γ-^{32}P]ATP was determined as previously described (Inesi et al., 1980).

Phosphoenzyme formation in the absence of Ca^{2+} was measured at equilibrium (Punzengruber et al., 1978) and in the transient state (Chaloub et al., 1979) for determination of phosphoenzyme levels and rate constants.

Protein concentrations were determined by the Lowry (1951) method.

Amino acid analysis was carried out on a 120C Beckman analyzer adapted for a single column. Identical samples were hydrolyzed in 6 N HCl at 110 °C for 24 h, or in mercaptoethanesulfonic acid as described by Penke et al. (1974). Cysteine was estimated by titration of sulfhydryl groups as described by Murphy (1976).

Free Ca^{2+} concentrations were estimated from total calcium and EGTA by computations (Fabiato & Fabiato, 1979) taking into account pH, Mg^{2+} concentration, and nucleotide concentration when present. The binding constant given by Schwartzenbach et al. (1957) was used for the Ca·EGTA complex.

Computations. Computations were carried out with the assistance of a North Star Horizon microcomputer, equipped with a 32K memory and dual floppy disk drives.

Reagents were obtained from commercial sources. The detergent dodecyloctaethylene glycol monoether (Nikkol, Japan) was obtained through the kind intervention of Professor Y. Tonomura.

Results

Calcium Binding to Purified ATPase. Both calcium binding measurements (Inesi et al., 1980) and the dependence of enzyme activity on Ca^{2+} concentration (de Meis & Hasselbach, 1971; The & Hasselbach, 1972; Vianna, 1975; Neet & Green, 1977) indicate that the high-affinity calcium binding observed in SR vesicles is a property of the ATPase. This enzyme constitutes the most prominent protein in SR vesicles, can be obtained in purified form, and is dissociated by strong detergents into polypeptide chains of M_r 102 000–120 000 (MacLennan et al., 1973; Allen & Green, 1978; Rizzolo et al., 1976).

Since interpretation of our experiments requires a precise stoichiometry of the binding sites with respect to the ATPase chains, we carried out measurements of calcium binding with purified ATPase in addition to SR vesicles. For these measurements, we used ATPase purified by the methods of MacLennan (1970) or Meissner et al. (1973). Electrophoretic patterns and specific activities of these preparations were comparable to those reported in the literature.

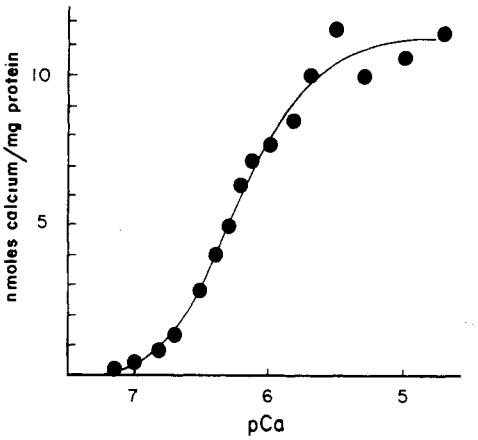


FIGURE 1: Calcium binding to ATPase purified according to MacLennan (1970). Binding was measured by equilibrium dialysis. the reaction medium consisted of 20 mM Mops (pH 6.9), 80 mM KCl, 5 mM $MgCl_2$, 45 μM $^{45}CaCl_2$, 10 mg of SR protein/mL, and various concentrations of EGTA to yield the desired pCa. The temperature was 25 °C.

A critical point for the satisfactory evaluation of site stoichiometry is an accurate determination of the protein concentration which is routinely measured by the Lowry method (1951). It was pointed out by Hardwicke & Green (1974) that the intensity of color development is 1.2 higher for unit dry weight of *delipidated* SR ATPase than for serum albumin which is used as a standard. Therefore, the values obtained by the Lowry method should be reduced accordingly. On the other hand, in amino acid analysis of our samples, we found a 10% excess of the total amino acid recovery over that expected from Lowry determinations of *lipid-bound* ATPase for which serum albumin was used as a standard without corrections. Therefore, we report our data in reference to protein weight units related to amino acid analysis. The amino acid composition of our ATPase samples is in satisfactory agreement with that reported in the literature for SR ATPase (Allen et al., 1980).

Titrations of high-affinity calcium binding of SR ATPase are shown in Figure 1. Maximal binding levels were found to be 9–11 nmol/mg of protein for the ATPase purified according to MacLennan (1970), 8–9 nmol/mg of protein for the ATPase purified according to Meissner et al. (1973), and 7–8 nmol/mg for native SR vesicles. In all cases, the maximal levels of calcium bound were related with a ratio of 2 (Table I) to the maximal levels of phosphoenzyme (Yamamoto & Tonomura, 1967; Makinose, 1969) obtained in the presence of ATP in steady-state conditions. However, the maximal levels of calcium bound were related with a ratio of 1 to the maximal number of sites available for phosphorylation with P_i (Masuda & de Meis, 1973) in the absence of Ca^{2+}, as determined in equilibrium conditions (Figure 2).

On the basis of these values, and consideration of the monomeric state of the enzyme (Brady et al., 1981), a stoichiometry of two calcium sites per polypeptide chain would account only for 46–66% (depending on a M_r of 102 000 or 120 000) of our preparation of the MacLennan (1970) ATPase. It is possible that partial denaturation and heterogeneities account for the remaining protein. Alternatively, it is possible that each polypeptide chain contains one calcium

Table I: Stoichiometry of Calcium and Phosphorylation Sites in the ATPase of SR Vesicles[a]

calcium sites (max no. derived from equilibrium expts)	7–8 nmol/mg of protein
phosphoenzyme (steady-state levels obtained with ATP in the presence of Ca^{2+})	3.5–4.0 nmol/mg of protein
phosphoenzyme sites (max no. derived from equilibrium expts with P_i in the absence of Ca^{2+})	7.7–8.9 nmol/mg of protein

[a] The experimental conditions for calcium binding and phosphorylation with P_i are described in the legends of Figures 2 and 4. Phosphorylation with ATP was obtained by incubation (15 s at 3 °C, or 50 ms at 25 °C) of 0.2–0.3 mg of protein with 0.1 mM [γ-^{32}P]ATP, in the presence of 50 μM $CaCl_2$, 80 mM KCl, 5 mM $MgCl_2$, and 20 mM Mops (pH 6.8) buffer. The reaction was quenched with acid and the phosphoenzyme measured as previously described (Inesi et al., 1980).

site, and two chains join to form one enzyme unit. At any rate, it is certain that within one operative enzyme unit, two interacting sites are occupied by calcium, while only one of two phosphate sites is phosphorylated by ATP in steady-state conditions. In addition to high-affinity sites, cation sites with intermediate ($K_d \simeq 5 \times 10^{-5}$ M) and low ($K_d \simeq 10^{-3}$ M) affinity are also found in SR vesicles (Inesi et al., 1980) and are retained by ATPase following purification.

Studies with Solubilized Enzyme Preparations. In all measurements of calcium binding to the ATPase enzyme assembled in native vesicles or reassembled in membranous structures following purification, we found that titration of high-affinity calcium sites exhibits a cooperative behavior. We have previously attributed this behavior to the interaction of two sites and fitted the titration curve with two constants, each constant being 1 order of magnitude lower or higher than the apparent affinity constant (Inesi et al., 1980). Our present experiments, yielding n_H values higher than 2 at alkaline pH (see below and Figure 4), indicate that higher order interactions may occur as the pH is increased above neutrality.

We then turned our attention to the enzyme in a solubilized form. For this purpose, we chose the detergent dodecyloctaethylene glycol monoether ($C_{12}E_8$), which is capable of solubilizing SR ATPase in a monometric and still active form (Dean & Tanford, 1978). However, at the high $C_{12}E_8$:protein ratios that are required for effective solubilization, we found intractable difficulties in maintaining the high protein concentrations required to detect calcium binding by equilibrium methods. Therefore, we resolved to measure Ca^{2+}-dependent ATPase activity as a parameter representative of calcium binding. It is demonstrated in Figure 3 that the Ca^{2+} concentration dependence of ATPase activity in the absence of $C_{12}E_8$ displays a cooperative behavior similar to that observed for calcium binding experiments. In fact, at alkaline pH, the n_H value for the Ca^{2+} concentration dependence of ATPase activity (Figure 3) is somewhat lower than that of equilibrium binding (Figure 4), while it is identical for both ATPase and binding at neutral pH. The higher cooperativity obtained for equilibrium binding at alkaline pH may reflect higher order chain interactions occurring with slower kinetics than the enzyme turnover (Roberts, 1977). The specific interaction involved in each transport cycle appears to involve only two sites.

A sharp change in the titration curve is obtained when the enzyme is solubilized in $C_{12}E_8$, as noted by Verjovski-Almeida & Silva (1981) with measurements of intrinsic fluorescence. We show in Figure 3 that in reaction mixtures containing 25

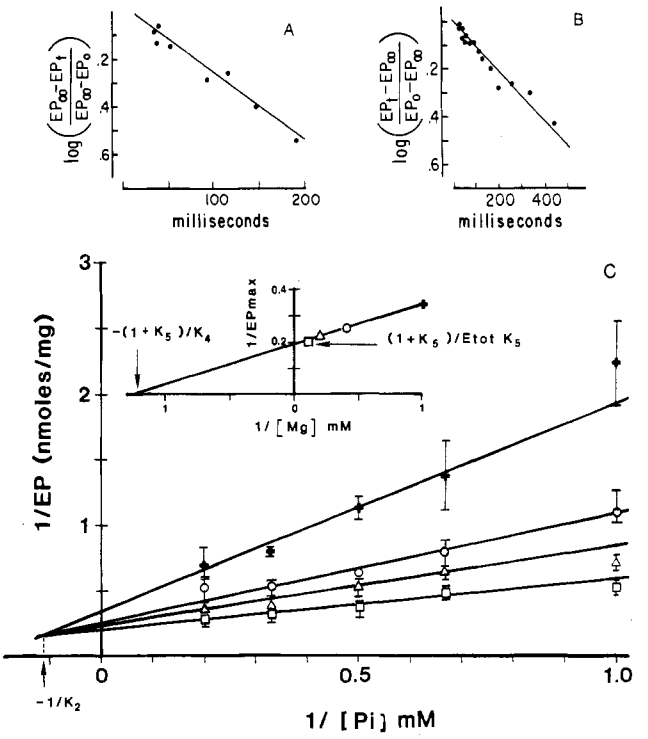
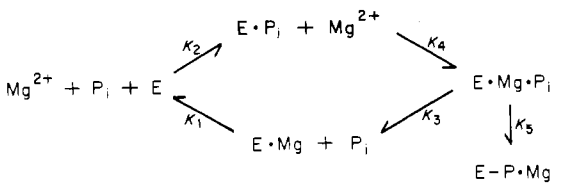


FIGURE 2: Kinetic and equilibrium studies on enzyme phosphorylation with P_i in the absence of Ca^{2+}. (A) Kinetic resolution of phosphoenzyme formation. The reaction mixture consisted of 50 mM Tris–maleate (pH 6.0), 10 mM $MgCl_2$, 2 mM [^{32}P]P_i, and 0.5 mg of SR protein/mL. The reaction was started by the addition of [^{32}P]P_i to SR, followed by acid quenching at serial times in a Dionex multimixing apparatus; the temperature was 25 °C. This type of experiment yields a $k^{obsd} = k_2 + k_{-2}$; k_{-2} is obtained directly in phosphoenzyme decay experiments, as in (B). (B) Kinetic resolution of phosphoenzyme decay. Phosphoenzyme was formed by preliminary equilibration of SR protein (1 mg/mL) with 50 mM Tris–maleate (pH 6.0), 10 mM $MgCl_2$, 0.5 mM EGTA, and 2 mM [^{32}P]P_i. ^{32}P-labeled phosphoenzyme decay was then started by a 13-fold dilution of the isotope with cold P_i, and serial samples were taken thereafter following acid quenching. Rapid mixing for dilution and quenching was obtained with the Dionex multimixing apparatus which is equipped with two mixing chambers and temperature control; the temperature was 25 °C. A value for k_{-2} was derived directly from the experiment. Therefore, $k_2 = 3.9$ s^{-1}, $k_{-2} = 2.4$ s^{-1}, and $K_5 = 1.6$ (at pH 6, and in the absence of K^+). Much faster kinetics are obtained at pH 7.0, in the presence of K^+. (C) Equilibrium levels of phosphoenzyme at increasing P_i concentrations in the presence of various Mg^{2+} concentrations. The reaction mixture consisted of 50 mM Tris–maleate, pH 6.0, 0.5 mM EGTA, 0.5 mg of SR protein/mL, and variable Mg^{2+} and [^{32}P]P_i concentrations. Incubation was for 5 min at 25 °C, followed by acid quenching. A parallel series of experiments was done at increasing Mg^{2+} concentrations in the presence of various P_i concentrations



as proposed by Punzengruber et al. (1978). The K_a values obtained from the equilibrium studies [as in (C)] are $K_1 = 120$, $K_2 = 110$, $K_3 = 455$, and $K_4 = 500$. While in previous studies (Punzengruber et al., 1978; Martin & Tanford, 1981) K_5 was derived by the ratio of an assumed number of phosphorylation sites to the experimental $E\text{–}P_{max}$ at equilibrium, we determined K_5 from k_5/k_{-5} which was obtained in kinetic experiments (A and B). The advantageous feature of the combined kinetic and equilibrium studies is the ability to estimate without assumptions the total number of reactive sites directly, as $E_{tot} = (1 + K_5)E\text{–}P_{max}/K_5$. The maximal number of phosphorylation sites estimated for the SR vesicles used in these studies was between 7.7 and 8.9 nmol/mg of protein.

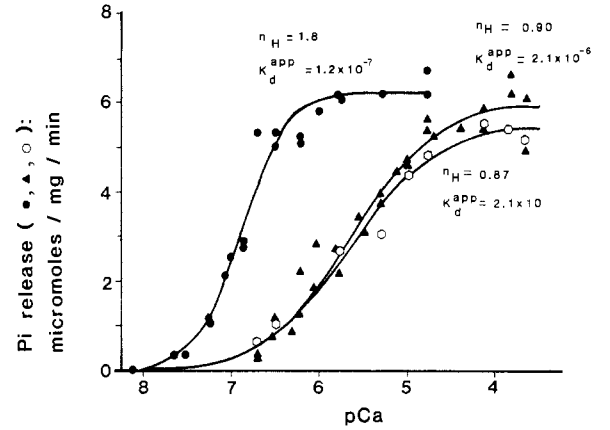


FIGURE 3: Effect of $C_{12}E_8$ on the Ca^{2+} concentration dependence of ATPase activity. The reaction mixture consisted of 10 mM *N*-2-(hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (Hepes), pH 7.5, 0.8 mM KCl, 10 mM $MgCl_2$, 50 μM $CaCl_2$, and various concentrations of EGTA. The protein concentration was 25 μg/mL in the absence (●) or in the presence of 0.2 (○) or 1.5 mM (▲) $C_{12}E_8$. Following 10-min incubation of the protein with detergent, the reaction was started with 5 mM ATP; the temperature was 25 °C.

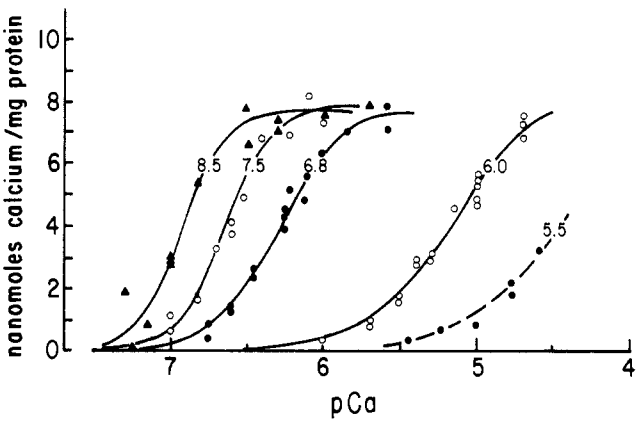


FIGURE 4: Effect of pH on calcium binding. The measurements were carried out following equilibration by the column chromatograph method. Buffer consisted of 10 mM Tris (pH 8.5) Hepes (pH 7.5), Mops (pH 6.8), or Mes (pH 6.1 and 5.5). In addition to buffer, the medium contained 10 mM $MgCl_2$, 80 mM KCl, 45 μM $^{45}CaCl_2$, and various concentrations of EGTA. The passive binding measured here was identical in the presence or in the absence of Ca^{2+} ionophores.

μg of protein/mL in the presence of 0.2–1.5 mM $C_{12}E_8$, the cooperative behavior of the ATPase dependence on the Ca^{2+} concentration is entirely lost. Furthermore, the Ca^{2+} concentration producing half-maximal activation is increased from 1.2×10^{-7} to 2.1×10^{-6} M. The latter value may be used directly to derive the intrinsic binding constant (K_1) for the calcium sites in the absence of cooperative interaction. The K_1 value is then 4.8×10^5 M^{-1} (at pH 7.5), which is approximately 1 order of magnitude lower than K^{app} (8.9×10^6 M^{-1}); on the other hand, the high-affinity constant (K_2) developed as a consequence of a cooperative interaction should be approximately 1 order of magnitude higher than K^{app}. It will be recalled that a similar relationship of constants was predicted by our analysis of cooperative binding at pH 6.8 (Inesi et al., 1980), yielding $K^{app} = 2.3 \times 10^6$ M^{-1} (observed), $K_1 = (1.2 \pm 0.3) \times 10^5$ M^{-1}, and $K_2 = (5 \pm 0.6) \times 10^7$ M^{-1}.

The Ca^{2+} concentration dependence of ATPase activity in the presence of $C_{12}E_8$ was previously studied by Møller et al. (1980). These authors obtained a composite curve with a cooperative component at low Ca^{2+} concentrations ($K_d^{app} \simeq 10^{-7}$ M), and a further activation at higher Ca^{2+} concentrations ($K_d^{app} \simeq 10^{-5}$ M). In light of our experience, this composite curve can be attributed to the coexistence of oligomeric and monomeric enzyme populations with the conditions used. It should be pointed out that optimal conditions for monomerization and total preservation of enzyme activity are obtained when a very low concentration of protein (25 μg/mL) is solubilized in a high detergent concentration (1 mM), without altering further the detergent:protein ratio by additional manipulations.

It is noteworthy that the Ca^{2+} concentration dependence of the solubilized ATPase did not change when the $C_{12}E_8$ concentration was increased from 0.2 to 1.5 mM (Figure 3). In fact, significant calcium binding to $C_{12}E_8$ was excluded by experiments on calcium titration of EGTA (Verjovski-Almeida & Silva, 1981).

The effect of detergent solubilization, and its interference with the cooperative behavior of the Ca^{2+} concentration dependence of ATPase, suggests that single polypeptide chains retain enzyme activity which is dependent on the occupancy of noninteracting calcium site(s).

pH Dependence of High-Affinity Calcium Binding to SR Vesicles. Our previous measurements of calcium binding (Inesi et al., 1980) were carried out at neutral pH. Since other reports indicated a pH dependence of calcium binding (Meissner, 1973; Verjovski-Almeida & de Meis, 1977; Beil et al., 1977), we have now studied the pH dependence of calcium binding by equilibrium measurements. We found that the apparent dissociation constant (K_d^{app}) is reduced by 2 orders of magnitude when the pH is raised from 5.5 to 8.5.

The experimental points in Figure 4 show an excellent resolution of high-affinity binding at alkaline and neutral pH, and no low-affinity binding is detected within 2 orders of magnitude in the Ca^{2+} concentration range. Therefore, the experimental points were fitted according to the Hill equation (1913):

$$[Ca_{bound}] = \frac{[Ca_{bound}^{max}][Ca^{2+}]^{n_H}}{K_H + [Ca^{2+}]^{n_H}} \quad (1)$$

where K_H (the Hill constant) is related to $\bar{Y}$ (the fractional saturation of binding sites) and n_H (the Hill coefficient) as

$$K_H = \frac{(1 - \bar{Y})[Ca^{2+}]^{n_H}}{\bar{Y}} \quad (2)$$

From the experimental data, it was then possible to estimate the three unknown values corresponding to $[Ca_{bound}^{max}]$, n_H, and K_H, using a nonlinear regression technique. Thereby satisfactory fits were obtained for curves obtained at pHs ranging from 8.5 to 6.1. At pH 5.5, a precise analysis was rendered difficult by interference of low-affinity binding at Ca^{2+} concentrations higher than 10^{-5} M. Therefore, only an approximate fitting to the first portion of the site's titration was drawn, in order to obtain an approximate value for K_d^{app} at this pH.

The estimated K_d^{app} values are 2.1×10^{-5} (pH 5.5), 7.6×10^{-6} (pH 6.0), 5.1×10^{-7} (pH 6.8), 2.2×10^{-7} (pH 7.5), and 1.2×10^{-7} M (pH 8.5).

In addition to an effect on the apparent affinity for Ca^{2+}, a rise in pH appears to enhance the cooperative character of the titration curve, and n_H values increase from 1.46 at pH 6.0, to 2.1 at pH 6.8, 2.95 at pH 7.5, and 3.3 at pH 8.5.

Effect of Temperature and Substrate (Nucleotide) Binding on Calcium Binding to Purified ATPase. It is known that certain functional and structural parameters of SR ATPase are highly temperature dependent. Nevertheless, when we compared equilibrium calcium binding to purified ATPase

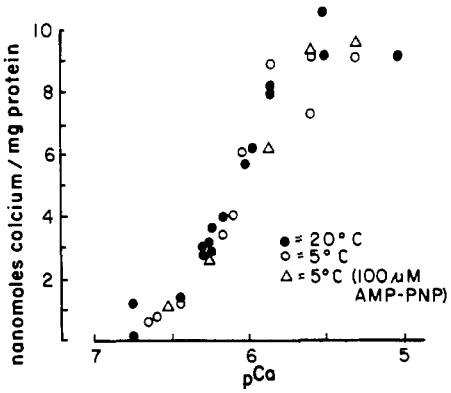


FIGURE 5: Calcium binding to purified ATPase: effect of temperature and AMP-P(NH)P. Equilibrium in chromatography columns. The medium consisted of 10 mM Mops (pH 6.9), 80 mM KCl, 10 mM $MgCl_2$, 45 μM $^{45}CaCl_2$, and various concentrations of EGTA. ATPase purified according to Meissner et al. (1973) was used in these experiments.

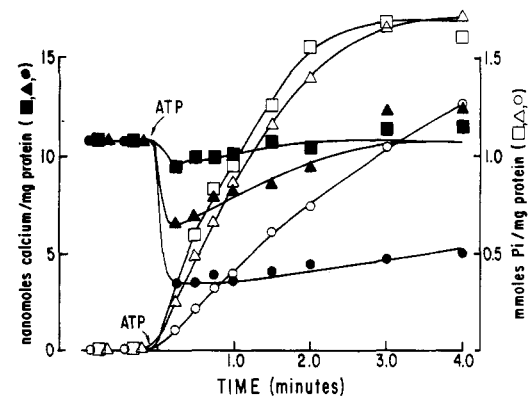


FIGURE 6: Calcium release and ATP hydrolysis (P_i production) by purified ATPase: effect of Me_2SO concentration. Calcium binding was measured by the filter method. ATP hydrolysis was determined at [^{32}P]P_i release from [γ-^{32}P]ATP. The reaction mixture consisted of 20 mM Mes (pH 6.1), 80 mM KCl, 5 mM $MgCl_2$, 50 μM $^{45}CaCl_2$, 10 mM [^{3}H]glucose, and 0.5 mg of ATPase/mL. At zero time, 1.0 mM ATP was added. ATPase purified according to MacLennan (1970) was used in this experiment; the temperature was 25 °C. Me_2SO concentrations were 0 (■, □), 20% (▲, △), and 30% (●, ○).

(membranous form) at 25 and 5 °C, we found no significant difference in the number of high-affinity sites or in the cooperative character of the binding titration (Figure 5). The apparent K_d could be somewhat reduced at the low temperature, since some uncertainty is introduced here by our neglect of the temperature dependence of the EGTA·Ca binding constant in the calculation of free Ca^{2+} concentration.

We also found no significant difference when binding was measured in the presence of AMP-P(NH)P (adenyl-5′-yl imidodiphosphate) (Figure 5), an ATP analogue that binds but does not phosphorylate the enzyme. It is noteworthy that although no effect of the nucleotide is observed on calcium binding in equilibrium conditions, it is possible that the nucleotide may influence the kinetics of the enzyme following calcium binding.

Effect of Substrate Utilization on Calcium Binding to Purified ATPase. Considering the lack of an AMP-P(NH)P effect on calcium binding to the high-affinity ATPase sites, it is of great interest to demonstrate an effect of ATP which, as opposed to AMP-P(NH)P, is utilized as a substrate to form a phosphorylated enzyme intermediate which then undergoes hydrolytic cleavage. This problem cannot be studied at equilibrium owing to the tendency of ATP to undergo nearly complete hydrolysis. Therefore, experimentation must be conducted in transient or steady-state conditions. Furthermore, a "leaky" ATPase preparation must be used in order to avoid interference of active transport with measurements of calcium binding. This type of experiment was conducted by Ikemoto (1975, 1976), who observed a release of bound calcium following ATPase phosphorylation with ATP, using an enzyme prepared by solubilization of native vesicles with Triton X-100. On the other hand, Takakuwa & Kanazawa (1979), and Dupont (1980), reported experiments indicating that the bound calcium is not released immediately upon phosphorylation but rather is retained in an occluded form which is not accessible from the outer or the inner side of the membrane. In this case, the occluded state would be prevalent in steady-state operation of the enzyme.

In our laboratory, we have repeatedly failed to demonstrate calcium release upon phosphorylation of SR ATPase prepared according to MacLennan (1970) or Meissner et al. (1973), independent of pH variations from 6.0 to 7.0. However, we reached a turning point in this difficulty when we added dimethyl sulfoxide (Me_2SO) to the reaction mixture. Me_2SO was first used by The & Hasselbach (1977), who found that the ATPase hydrolytic activity is inhibited by this solvent, while the phosphorylated enzyme intermediate is maintained at maximal levels. Shigekawa & Akowitz (1979) reported that Me_2SO specifically inhibits hydrolysis of the ADP-insensitive phosphoenzyme. More recently, de Meis et al. (1980) demonstrated that the equilibrium of the P_i reaction with the enzyme is shifted greatly in favor of phosphoenzyme formation. Therefore, we reasoned that under the influence of this solvent the lifetime of a low-affinity species of the phosphoenzyme may be prolonged, thereby permitting detection of calcium release upon enzyme phosphorylation with ATP. We also chose a rather low pH (6.1), which also favors formation of phosphoenzyme from enzyme and P_i (Masuda & de Meis, 1973), e.g., reversal of the hydrolytic reaction. A first series of experiments was conducted by isotopic tracer and filtration methods. Time resolution was rendered possible by the slow kinetics conferred on the system by low pH and Me_2SO.

It is shown in Figure 6 that at zero time approximately 10 nmol of calcium is bound per mg of ATPase protein, demonstrating saturation of the high-affinity sites in accordance with the equilibrium measurements shown in Figure 1. A barely detectable amount of calcium is released upon addition of ATP in the absence of Me_2SO. On the other hand, significant calcium release (up to 60–70% of the calcium bound) is detected in the presence of Me_2SO. A parallel reduction of P_i production is also observed, in accordance with the inhibition of hydrolytic activity produced by increasing concentrations of Me_2SO. It should be pointed out that at pH 6.1 the hydrolytic activity of the ATPase prepared according to MacLennan (1970) is 70% lower than that at pH 7.5.

An important feature of these experiments is that calcium binds again to the ATPase when ATP is hydrolyzed. In fact, the duration of release is proportional to the concentration of ATP added to the reaction mixture. This interdependence is better established in experiments demonstrating a time relationship between formation of the phosphoenzyme and calcium release, followed by rebinding of calcium upon phosphoenzyme hydrolysis and P_i cleavage (Figure 7). It is clear that release and rebinding of calcium are due to changes in affinity of the binding site, related to phosphorylation and dephosphorylation of the enzyme.

Besides ATP, other substrates known to phosphorylate ATPase, such as ITP, *p*-nitrophenyl phosphate, and acetyl phosphate, cause calcium release (Figure 8). Some substrates such as *p*-nitrophenyl phosphate and acetyl phosphate produce calcium release at lower rates than the nucleotides, probably

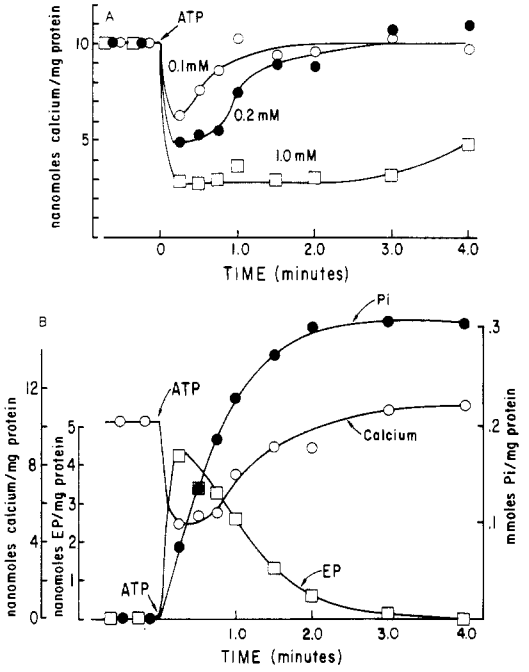


FIGURE 7: (A) Duration of Ca^{2+} release and concentration of ATP. (B) Calcium release, formation of phosphorylated enzyme intermediate, and P_2 liberation following addition of ATP in the presence of 30% Me_2SO. Calcium bound to ATPase was measured by the filter method. ATP (200 μM) was added at zero time. Phosphorylation of the enzyme and P_i production were measured with the aid of [γ-^{32}P]ATP, in conditions identical with those used for calcium measurements. Conditions were as described for Figure 6.

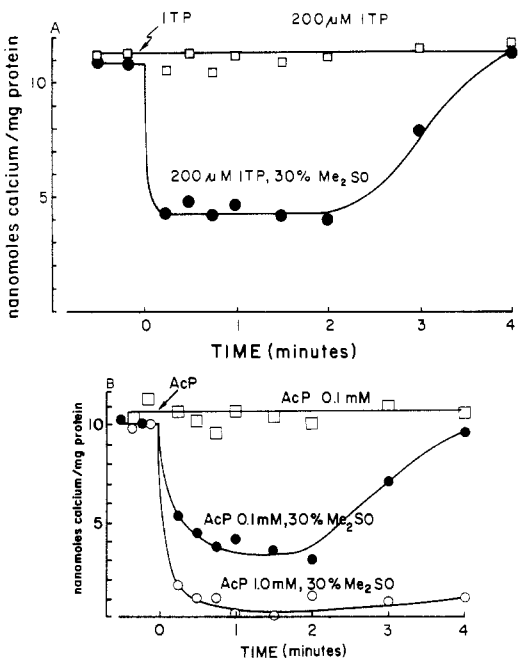


FIGURE 8: Calcium release from ATPase following addition of various substrates. Calcium bound to ATPase was measured by the filter method. At time zero, various substrates were added as indicated, in the presence or in the absence of Me_2SO. Reaction mixture was as described in Figure 6; the temperature was 25 °C. ATPase was purified according to MacLennan (1970).

due to the slower rates of enzyme phosphorylation. ADP does not phosphorylate the enzyme, nor does it cause calcium release.

In all cases, we found Me_2SO to be necessary to detect the release phenomenon upon enzyme phosphorylation. We found that 30% dimethylformamide permits detection of calcium release as well but also causes some leak of protein through the filters used to separate the ATPase from the medium. No significant calcium release could be detected in the presence of 30% glycerol.

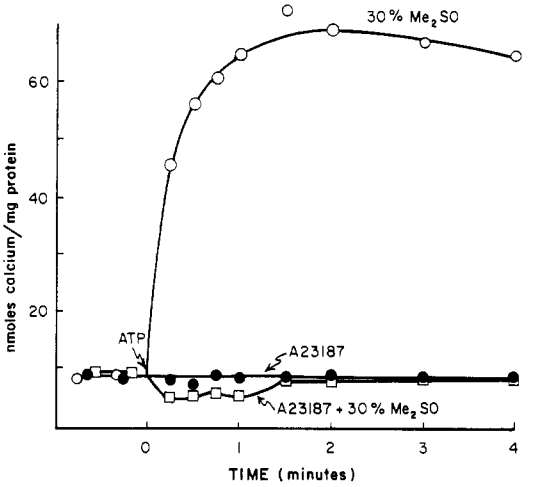


FIGURE 9: Effect of concentrations of Me_2SO and of the ionophore A-23187 on SR vesicles. Calcium uptake (net transport) and binding were measured as explained for Figure 6. In these experiments, native SR vesicles were used instead of purified ATPase. ATP (200 μM) was added at zero time in the presence of 30% Me_2SO (○, □) and/or 16.7 μM ionophore A-23187 (●, ■).

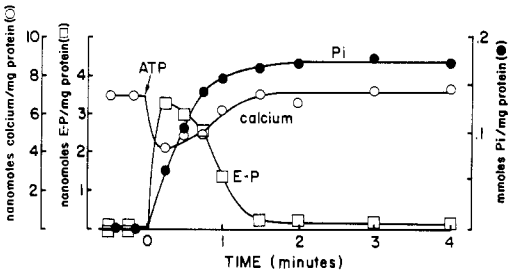


FIGURE 10: Calcium release, phosphoenzyme formation, and P_i release in SR vesicles following addition of ATP in the presence of 30% Me_2SO and ionophore A-23187. Measurements were as described for the experiment in Figure 7. In this experiment native SR vesicles were used instead of purified ATPase, and 17.6 μM A-23187 was added to make the vesicles leaky.

Effect of Enzyme Phosphorylation on Calcium Binding to Leaky SR Vesicles. To exclude the possibility that the observed calcium release, or even the Me_2SO requirement for detection of this phenomenon, may be a peculiar feature of the ATPase modified by the deoxycholate (DOC) purification procedure (MacLennan, 1970; Meissner et al., 1973), we repeated the release experiments with native SR vesicles. To avoid calcium accumulation in the lumen of the vesicles and to permit calcium release into the medium, we made the vesicles leaky by adding the ionophore A-23187 (Scarpa et al., 1972). It is shown in Figure 9 that even in this case Me_2SO is required for detection of the release phenomenon. On the other hand, in the absence of A-23187, the vesicles sustain active transport even in the presence of Me_2SO, demonstrating that the native state of the ATPase enzyme is still present.

The time relationship between enzyme phosphorylation and calcium release followed by hydrolytic cleavage of the enzyme and rebinding of calcium to the ATPase in native residues is shown in Figure 10.

Spectrophotometric Measurements of Ca^{2+} Release. In addition to measurements by isotopic tracer and filtration methods, Ca^{2+} transients were also measured with the aid of metallochromic indicators. Arsenazo III was chosen as the indicator, in analogy to the experiments reported by Ikemoto (1976). As the indicator is very sensitive to minimal changes

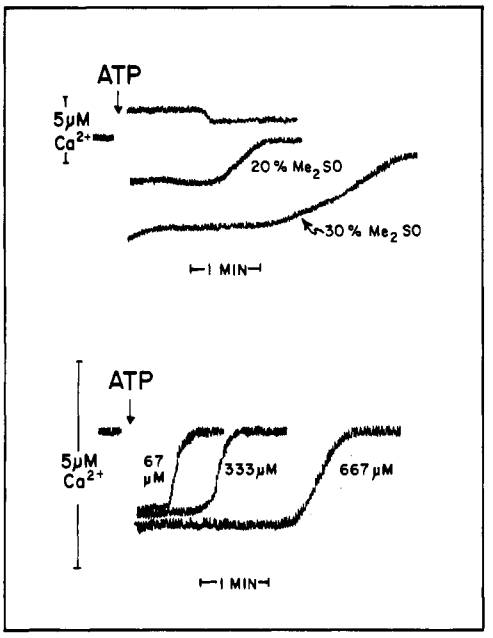


FIGURE 11: Spectrophotometric determination of calcium release from ATPase following addition of ATP. (Top) Effect of Me_2SO concentration. The reaction mixture consisted of 0.4 mg of ATPase (MacLennan, 1970) per mL, 20 mM Mes (pH 6.1), 45 μM $CaCl_2$, 80 mM KCl, 5 mM $MgCl_2$, 50 μM arsenazo III, and various concentrations of Me_2SO. ATP (333 μM) was added at time zero. The base line is normalized to the absorption levels obtained after exhaustion of ATP, and the specific absorption change due to the calcium standard is normalized for the different absorption noted at different Me_2SO concentrations; the temperature was 25 °C. (Bottom) ATP concentration dependence of calcium release from ATPase. Conditions as in (A), except for an ATPase concentration of 0.71 mg/mL and a Me_2SO concentration of 20%.

(micromolar) of the Ca^{2+} concentration, care must be taken to distinguish artifacts due to Ca^{2+} binding to ATP. In fact, a rapid signal corresponding to Ca^{2+} binding is obtained upon addition of ATP, independent of whether ATPase is present or not in the medium. If ATPase is present in the medium, this signal is slightly greater (Figure 11 top) as if the vesicular aggregates of purified enzyme retained a slight capacity for active transport, or another binding site were uncovered by phosphorylation. This small amount of calcium is then released following complete utilization of ATP in the medium. It should be pointed out that neither the ATP artifact nor this slight calcium uptake is noted when measurements are made with radioactive isotope and filtration methods (Figures 4–6). At any rate, and most importantly, no calcium release is detected by either isotope or spectroscopic method when ATP is added to the enzyme in the absence of Me_2SO.

A quite different pattern is obtained in the presence of Me_2SO. In this case, Ca^{2+} release from the enzyme upon addition of ATP can be easily observed as a prominent phenomenon which obscures any small binding that may occur simultaneously (Figure 11, top). In analogy to the experiments with radioactive isotope, calcium binds again to the enzyme when the ATP of the medium is completely utilized and the phosphoenzyme decays. It is shown in the lower portion of Figure 11 that the duration of the release phenomenon is proportional to the ATP concentration.

Release phenomena similar to those obtained with ATP were observed with other substrates such as ITP, *p*-nitrophenyl phosphate, and acetyl phosphate, using the spectrophotometric method of detection. In all cases, addition of Me_2SO was required for detection of the release. Although ethanol and dimethylformamide were also effective, no significant release was observed in the presence of dioxane or ethylene glycol,

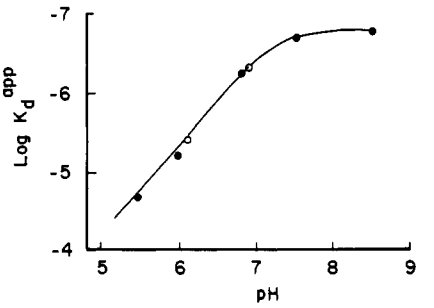


FIGURE 12: pH dependence of the K_d for calcium binding to high-affinity ATPase sites. Data were obtained as described in Figure 4. ATPase purified according to MacLennan (1970) (○) or native SR vesicles (●) were used for these determinations.

or in the absence of organic solvents.

Discussion

Calcium Binding. A first point of interest is the stoichiometry of the high-affinity calcium sites with respect to the phosphorylation sites and the ATPase chains. Our present measurements yield maximal levels of 9–11 nmol of calcium bound per mg of protein of purified ATPase. We have previously reported that two calcium ions are bound by one enzyme unit phosphorylated with ATP in steady-state conditions (Inesi et al., 1980). We now confirm such a ratio but find a ratio of 1 between calcium sites, and sites available for phosphorylation with P_i in the absence of Ca^{2+} (Masuda & de Meis, 1973) in equilibrium conditions (Table I). We also find that the cooperative character of calcium binding previously noted with native vesicles is obtained with purified ATPase, and for the Ca^{2+} concentration dependence of enzyme activity as well. The latter behavior is abolished by protein dissociation with $C_{12}E_8$.

These findings suggest that (a) one polypeptide chain (M_r 100 000) contains one calcium and one phosphorylation site, (b) one enzyme unit includes two polypeptide chains, and (c) the cooperative interaction is abolished when the chains are dissociated by $C_{12}E_8$. Since the presence of inactive protein cannot be excluded with certainty, it is also possible that one chain contains two calcium sites, and the cooperative appearance of the titration curve is abolished by additional effects of the detergent, concomitant with membrane dissociation.

Our studies demonstrate with certainty that within one native enzyme unit two interacting sites bind calcium, while only one of two phosphorylation sites is phosphorylated by ATP in steady-state conditions. The relevance of such a site stoichiometry to calcium transport is underlined by an optimal ratio of 2 between calcium transport and ATP hydrolysis (Hasselbach, 1964).

An interesting feature of the binding mechanism is its pH dependence. Such a dependence is formalized by the equation derived by Wyman (1964) for oxygen binding to hemoglobin:

$$\left[\frac{\delta \log P}{\delta(\mathrm{pH})}\right]_{\bar{Y}} = \left(\frac{\delta[\mathrm{H}^+]}{\delta \bar{Y}}\right)_{\mathrm{pH}} \qquad (3)$$

where P is the oxygen tension and $\bar{Y}$ the fractional saturation of binding sites. In our case, eq 3 can be utilized in the form

$$\frac{\delta \log K_d^{app}}{\delta(\mathrm{pH})} = -\Delta[\mathrm{H}^+] \qquad (4)$$

thereby generating the plot in Figure 12. Since the slope of the linear portion of the plot is approximately 1, it is apparent that one H^+ is liberated per calcium bound in the 5.5–6.8 pH

range. As the pH is increased, the H^+:Ca^{2+} ratio is reduced to reach 0 at pH 8.5. This behavior is consistent with the presence of one ionizable group at the calcium site, undergoing dissociation with a pK^{app} of 7.3. As for the identity of the ionizable group, the presence of a carboxyl is indicated by the reactivity of the calcium site to dicyclohexylcarbodiimide (DCC) (Pick & Racker, 1979; Murphy, 1981). If this is the dissociating group, the rather high pK value suggests a hydrophobic environment.

The stoichiometry of one dissociating group per site is in agreement with our previous direct measurements of H^+ release upon calcium binding and transport cycles (Chiesi & Inesi, 1980). It is noteworthy that there is no correlation between the number of carboxyl ligands and the affinity for calcium in calcium binding proteins of known structure (Kretsinger, 1976). In fact, main-chain carbonyl and water oxygens are most often involved as additional ligands. It should be pointed out that a change in the pK of the dissociating group or of the pH in the medium could play an important role in modulation of the calcium site affinity in the enzyme cycle, since Ca^{2+} exchange with H^+ is an integral part of the active transport mechanism.

Involvement of the Calcium Sites in Active Transport. It has been suggested that the mechanism of calcium transport and release against a concentration gradient includes a change in orientation and a reduction in affinity of the calcium binding sites following ATPase phosphorylation with ATP (de Meis & Vianna, 1979; Inesi, 1979; Tada et al., 1978). Rapid occlusion of the calcium sites from the outer surface of the membrane into a position not exposed to the outer medium was demonstrated unambiguously by rapid quench experiments Kurzmack et al., 1977; Verjovski-Almeida et al., 1978; Chiesi & Inesi, 1979; Dupont, 1980). Furthermore, a reduction in binding affinity was demonstrated by monitoring the occupancy of the calcium sites in spin-labeled SR in the presence of Triton X-100 (Coan et al., 1979). More directly, Ikemoto (1975, 1976) observed a reduction in affinity of the binding sites and release of bound calcium upon phosphorylation of ATPase prepared with Triton X-100. On the contrary, Takakuwa & Kanazawa (1979), and Dupont (1980), using other types of enzyme preparations, obtained evidence of retention of calcium in an occluded state upon phosphorylation of the enzyme with ATP.

In the experiments reported here, we found that detection of Ca^{2+} release upon phosphorylation of ATPase prepared according to MacLennan (1970), or of "leaky" SR vesicles, requires the presence of Me_2SO. This suggests that Me_2SO prolongs the lifetime of the phosphoenzyme following Ca^{2+} release, thereby rendering possible detection of net release before the phosphoenzyme turns over and binds more Ca^{2+}. This can be better explained by the following reaction scheme

$$Ca_2{\cdot}E + ATP \rightleftharpoons Ca_2{\cdot}E{\sim}P(\cdot ADP) \quad (a)$$

$$Ca_2{\cdot}E{\sim}P(\cdot ADP) \rightleftharpoons Ca_2{\cdot}{}^*E{-}P + ADP \quad (b)$$

$$Ca_2{\cdot}{}^*E{-}P \rightleftharpoons {}^*E{-}P + 2Ca^{2+} \quad (c)$$

$${}^*E{-}P \rightleftharpoons E + P_i \quad (d)$$

$$E + 2Ca^{2+} \rightleftharpoons Ca_2{\cdot}E \quad (e)$$

where E and *E correspond to enzyme states with high-affinity sites and low-affinity sites, respectively. Since it is known that step a (Froehlich & Taylor, 1975; Verjovski-Almeida et al., 1978) is not rate limiting, we conclude that steps b or c are rate limiting[1] in native enzyme preparations, while step d which is normally fast becomes rate limiting in the presence of Me_2SO. Therefore, the prevalent steady-state species in the two sets of conditions are [$Ca_2{\cdot}E{\sim}P + Ca_2{\cdot}{}^*E{-}P$] or [*E–P], respectively. It should be pointed out that in the former case, the entire population of phosphoenzyme is reactive to ADP by equilibration of $Ca_2{\cdot}E{\sim}P$ with $Ca_2{\cdot}{}^*E{-}P$, as demonstrated by ATP synthesis upon addition of high [Ca^{2+}] and ADP to *E–P (Knowles & Racker, 1975; de Meis & Tume, 1977). Therefore, this corresponds to the experimental definition of "occluded" state as observed by Takakuwa & Kanazawa (1979), and Dupont (1980).

It seems likely that the slow Ca^{2+} release (steps b and c) is related to a transmembrane translational process. At any rate, the effect of Me_2SO can be attributed to displacement of the equilibrium of step d in favor of phosphoenzyme formation from enzyme and P_i (de Meis et al., 1980), as demonstrated by inhibition of P_i production (Figure 7). Therefore, *E–P concentration is permitted to build up, and Ca^{2+} release can be detected. On the other hand, it is apparent that detection of Ca^{2+} release in the presence of Triton X-100 is due to a direct accelerating effect of the detergent on steps b and c, since no inhibition of P_i production is observed in this case.

It is then clear that a change in the orientation and reduction in the affinity of the calcium sites can be identified with the mechanistic step utilizing free energy for vectorial transport of Ca^{2+} against a concentration gradient. Calcium and phosphorylation sites maintain a two-way relationship, inasmuch as occupation of the calcium sites is required for activation of the catalytic site, and phosphorylation of the catalytic site with ATP causes a change in the orientation and a reduction in the affinity of the calcium sites. In the normal operation of the pump, inward calcium release from the phosphorylated enzyme is a slow step rapidly followed by phosphoenzyme cleavage. Our observations indicate also that one group, dissociating with $pK^{app} = 7.3$, participates as a ligand for calcium complexation at each site.

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[1] After submission of this manuscript, we were kindly shown a manuscript by Y. Nakamura and Y. Tonomura, indicating that conversion of the ADP-sensitive to the ADP-insensitive phosphoenzyme (step 2 in our short scheme) is a slow step.

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Stoichiometry of H^+-Linked Dopamine Transport in Chromaffin Granule Ghosts†

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ABSTRACT: A proton-translocating adenosinetriphosphatase in adrenal medullary chromaffin granule ghosts can generate either a membrane potential (inside positive) or a pH gradient (inside acid). Dopamine uptake occurs in response to both the membrane potential and the pH gradient. The natural logarithm of the dopamine concentration gradient [ln (D_{in}/D_{out})] is linearly related to the membrane potential with a slope of $F/(RT)$. This dependence is not affected by the pH of the medium. ln (D_{in}/D_{out}) is linearly dependent on ln ($[H^+]_{in}/[H^+]_{out}$) with a slope of 2. These results indicate that dopamine is taken up via an exchange diffusion or antiport mechanism. The stoichiometry of this exchange is two H^+/dopamine cation and is independent of pH.

Catecholamines in the adrenal medulla are stored at high concentration (0.55 M) in the chromaffin granules. Consequently, the chromaffin granule membrane must maintain an enormous catecholamine concentration gradient. Catecholamine accumulation into the granules is driven by an inwardly directed proton-translocating adenosinetriphosphatase (ATPase).[1] Protons are pumped into the granules and then exchanged for catecholamines via an antiport or exchange dif-

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[1] Abbreviations used: ATPase, adenosinetriphosphatase; ATP, adenosine 5′-triphosphate; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)-phenyl hydrazone; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; ΔpH, transmembrane pH gradient; $\Delta\psi$, transmembrane electrical potential gradient; D_{in}/D_{out}, transmembrane dopamine concentration gradient.

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