Stoichiometry of Tight Binding of Magnesium and Fluoride to Phosphorylation and High-Affinity Binding of ATP, Vanadate, and Calcium in the Sarcoplasmic Reticulum Ca^{2+}-ATPase†

Takashi Daiho,‡ Tatsuya Kubota,‡,§ and Tohru Kanazawa*,‡

Department of Biochemistry and The Third Department of Internal Medicine, Asahikawa Medical College, Asahikawa 078, Japan

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ABSTRACT: We previously showed that, when the purified Ca^{2+}-ATPase from sarcoplasmic reticulum (SR) is treated with fluoride (F^-) in the presence of Mg^{2+}, a complete inactivation of the enzyme is induced by tight binding of approximately 2 mol of Mg^{2+} and 4 mol of F^- to the catalytic site per mole of phosphorylation site [Kubota, T., Daiho, T., & Kanazawa, T. (1993) *Biochim. Biophys. Acta 1163*, 131–143]. Contradictorily, on the basis of the postulated content of the Ca^{2+}-ATPase in F^--treated SR vesicles, Coll and Murphy [(1992) *J. Biol. Chem. 267*, 21584–21587] suggested that each inactivated enzyme contains one tightly-bound Mg^{2+} and two tightly-bound F^-. The present study has been made to resolve this conflict. The contents of phosphorylation site, high-affinity ATP-binding site, high-affinity vanadate-binding site, and high-affinity Ca^{2+}-binding site in the SR vesicles used were 3.33 ± 0.06, 3.54 ± 0.12, 3.34 ± 0.04, and 6.98 ± 0.16 nmol/mg, respectively. When the vesicles were incubated with F^- in the presence of Mg^{2+}, the Ca^{2+}-ATPase was inactivated progressively. After removal of unbound Mg^{2+} and F^- by gel filtration, tightly-bound Mg^{2+} and F^- were determined by use of an atomic absorption spectrophotometer and a F^--selective electrode. A linear relationship existed between the extent of the enzyme inactivation and the contents of the tightly-bound ligands. The contents of tightly-bound Mg^{2+} and F^- in the fully inactivated vesicles were 6.65 and 12.6 nmol/mg, respectively. The same stoichiometry was obtained with another preparation of SR vesicles. These results demonstrate that tight binding of 2 mol of Mg^{2+} and 4 mol of F^- per mole of phosphorylation site, per mole of high-affinity ATP- or vanadate-binding site, and per 2 mol of high-affinity Ca^{2+}-binding site leads to a complete inactivation of the Ca^{2+}-ATPase in SR vesicles.

The Ca^{2+}-ATPase of skeletal muscle SR[1] is a membrane-bound enzyme that catalyzes ATP-driven Ca^{2+} transport (Hasselbach & Makinose, 1961; Ebashi & Lipmann, 1962). This enzyme consists of a single type of polypeptide chain with M_r 110 331, and its primary structure has entirely been revealed (Brandl et al., 1986). The enzyme has one high-affinity ATP-binding site and two high-affinity Ca^{2+}-binding sites per polypeptide chain. When both ATP and Ca^{2+} bind to the respective sites, the γ-phosphoryl group of ATP is transferred to Asp-351 of the enzyme to form an EP intermediate (Makinose, 1967; Yamamoto & Tonomura, 1967; Degani & Boyer, 1973; Bastide et al., 1973; Allen & Green, 1976). This EP can also be formed from P_i in the presence of Mg^{2+} and absence of Ca^{2+} by reversal of the catalytic cycle (Kanazawa & Boyer, 1973; Masuda & de Meis, 1973). Although the reaction sequence in the catalytic cycle has been mostly revealed by a number of kinetic and enzymatic studies, the detailed chemical and structural events in the catalytic site associated with the transport reaction remain largely unknown. One approach to this subject is to explore the reagents which can specifically bind to the catalytic site.

From this point of view, we previously demonstrated that Mg^{2+} and F^- did bind tightly to the catalytic site when the purified SR Ca^{2+}-ATPase was treated with F^- in the presence of Mg^{2+} and that this binding resulted in a complete inactivation of the enzyme (Kubota et al., 1993). These findings are consistent with those obtained with SR vesicles by Murphy and Coll (1992a,b). Since our results with the purified enzyme showed that the contents of tightly-bound Mg^{2+} and F^- in the fully inactivated enzyme were 9.7 and 15.5 nmol/mg, respectively, and that the content of phosphorylation site in the purified enzyme used was 4.2 nmol/mg, we concluded that tight binding of 2 mol of Mg^{2+} and 4 mol of F^- per mole of phosphorylation site leads to this enzyme inactivation.

On the other hand, Coll and Murphy (1992) showed with SR vesicles that the contents of tightly-bound Mg^{2+} and F^- in the F^--treated vesicles were 4.1 and 9.4 nmol/mg, respectively. On the assumption that the content of phosphorylation site (or the content of Ca^{2+}-ATPase) in the vesicles used is 4–5 nmol/mg, they deduced that each inhibited enzyme contains one tightly-bound Mg^{2+} and two tightly-bound F^-, although the content of phosphorylation site (or the content of Ca^{2+}-ATPase) in the vesicles was not actually determined. Thus, their conclusion contrasts sharply with ours. This discrepancy prompted us to determine the stoichiometry of tight binding of Mg^{2+} and F^- to high-affinity binding of ATP, vanadate, and Ca^{2+} as well as to phosphorylation in SR vesicles, since this stoichiometry most likely reflects structural and functional features of the catalytic site.

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* Address correspondence to this author at the Department of Biochemistry.

‡ Department of Biochemistry.

§ The Third Department of Internal Medicine.

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[1] Abbreviations: SR, sarcoplasmic reticulum; EP, phosphoenzyme; MOPS, 3-(*N*-morpholino)propanesulfonic acid; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; pCa, negative logarithm of molar Ca^{2+} concentration.

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The results obtained give evidence that tight binding of 2 mol of Mg^{2+} and 4 mol of F^- to the catalytic site per mole of phosphorylation site, per mole of high-affinity ATP- or vanadate-binding site, and per 2 mol of high-affinity Ca^{2+}-binding site leads to a complete inactivation of Ca^{2+}-ATPase in SR vesicles.

EXPERIMENTAL PROCEDURES

Preparation of SR Vesicles. SR vesicles were prepared from rabbit skeletal muscle as described previously (Kanazawa et al., 1971) with some modifications. Dorsal, psoas, and hind leg white muscles were homogenized with 4 volumes of 0.1 M NaCl and 10 mM MOPS–Tris (pH 7.0) in a Waring blender for 20 s four times at 5-s intervals. The homogenate was centrifuged at 8300*g* for 20 min. The supernatant was centrifuged at 12000*g* for 30 min to remove mitochondria. The resulting supernatant was centrifuged at 53000*g* for 40 min. The precipitate was suspended in 40 volumes of 0.6 M KCl and 5 mM Tris maleate (pH 6.5) and stirred gently for 40 min. The suspension was centrifuged at 125000*g* for 45 min, and the precipitate was washed once with a solution (solution A) containing 0.1 mM $CaCl_2$, 0.1 M KCl, 0.1 M sucrose, and 30 mM MOPS–Tris (pH 7.0). All the above procedures were carried out at 4 °C. The SR vesicles thus prepared were suspended in solution A and stored at −80 °C. These modifications brought about a substantial improvement in the content of the Ca^{2+}-ATPase in our preparations [cf. Suzuki et al. (1990)].

Treatment of SR Vesicles with F^-. The KF solution used was preincubated with 1 mM deferoxamine, a potent Al^{3+}-chelating agent (Ackrill & Day, 1984), to prevent a possible fluoroaluminate-induced inhibition of the SR Ca^{2+}-ATPase (Troullier et al., 1992). The SR vesicles (2.2 mg/mL) were preincubated at 25 °C for 1 h in a medium containing 1.1 mM deferoxamine, 5.5 μM $CaCl_2$, 110 mM KCl, 22% (v/v) DMSO, 22% (v/v) glycerol, and 44 mM imidazole hydrochloride (pH 7.5). A 0.07 volume of a solution containing EGTA and $MgCl_2$ was added to the mixture to give 1.02 mM EGTA and 10.2 mM $MgCl_2$, unless otherwise stated. The mixture was further preincubated for 5 min. The treatment of the vesicles with F^- was started at 25 °C by adding a 0.02 volume of the KF solution. The resulting mixture had a final composition of 2 mg of SR vesicles/mL, 1.0 mM KF (0.65 mM F^-), 10 mM $MgCl_2$ (9 mM Mg^{2+}), 1 mM EGTA, 5 μM $CaCl_2$, 1 mM deferoxamine, 20% (v/v) DMSO, 20% (v/v) glycerol, 0.1 M KCl, and 40 mM imidazole hydrochloride, unless otherwise stated. The reaction was quenched by diluting the mixture 1.7 times with an ice-cold solution containing various concentrations of $CaCl_2$ (100 μM Ca^{2+} at a final concentration), 0.1 M KCl, 0.1 M sucrose, and 30 mM MOPS–Tris (pH 7.0), and the mixture was centrifuged. The pellet was washed twice with solution A by centrifugation and then suspended in solution A. The efficiency of this quenching method had been ascertained by our previous observations (Kubota et al., 1993).

Tight Binding of Mg^{2+} and F^-. The F^--treated SR vesicles obtained as above were further incubated at 25 °C for 1 h in a medium containing 5 μM A23187, 10 mM EDTA, 0.1 M KCl, and 30 mM MOPS–Tris (pH 7.0). The vesicles were then applied at 4 °C to a coarse Sephadex G-50 column (1.5 × 25 cm) equilibrated with 2 mM EDTA, 0.1 M KCl, and 5 mM MOPS–Tris (pH 7.0), and the vesicles eluted were subjected to the determination of tightly-bound Mg^{2+} and F^-.

For the determination of tightly-bound Mg^{2+}, the suspension of the vesicles was applied to a centrifuge column (5-mL disposable syringe filled with coarse Sephadex G-50) equilibrated with deionized water at 4 °C. Magnesium bound to the eluted vesicles was extracted with 0.5 N HNO_3, and the concentration of magnesium in the extract was measured by use of an atomic absorption spectrophotometer as described previously (Kubota et al., 1993). For the assessment of magnesium contamination, in the above gel filtration (1.5 × 25 cm column) several fractions were collected before vesicles were eluted. These fractions were subjected to the centrifuge column and magnesium extraction in the same way as described above. The content of tightly-bound Mg^{2+} was obtained by subtracting the mean of the contents of magnesium [0.37 ± 0.11 nmol/mg (n = 7)] in the extracts from these control samples.

For the determination of tightly-bound F^-, fluoride was extracted by incubating the vesicles in a boiling water bath for 10 min. The sample was then centrifuged to remove insoluble materials, and DMSO and KNO_3 were added to the supernatant to give 20% (v/v) and 0.1 M, respectively. The pH of the sample was adjusted to 3.48 with citric acid, and the concentration of F^- was measured by use of a fluoride-selective electrode as described previously (Kubota et al., 1993).

Ca^{2+}-ATPase Activity. The total ATPase activity was measured at 25 °C in a mixture containing 10 μg of SR vesicles/mL, 0.2 mM [γ-^{32}P]ATP, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.4 mM EGTA, 2 μM A23187, 0.1 M KCl, and 30 mM MOPS–Tris (pH 7.0). The Ca^{2+}-ATPase activity was obtained by subtracting the Ca^{2+}-independent ATPase activity (which was measured in the presence of 5 mM EGTA without added $CaCl_2$, otherwise as above) from the total ATPase activity.

Content of Phosphorylation Site. The content of phosphorylation site in the SR vesicles was determined with [γ-^{32}P]ATP essentially according to the method of Barrabin et al. (1984), as described previously (Kubota et al., 1993).

ATP Binding. The SR vesicles (0.1 mg/mL) were incubated at 25 °C for 5 s in a medium containing 7.3–126 μM [α-^{32}P]ATP, 0.1 M KCl, 30 mM MOPS–Tris (pH 7.0), and others as described in figure and table legends, and the amount of bound [α-^{32}P]ATP was determined by filtration on a membrane filter as described previously (Kubota et al., 1993). The content of bound [α-^{32}P]ATP was obtained by subtracting the radioactivity on the filter through which the same solution without the vesicles was filtered.

Vanadate Binding. The SR vesicles (0.2 mg/mL) were incubated at 25 °C for 20 min in a medium containing 0.35–46.8 μM vanadate, 2 mM EGTA, 40% (v/v) DMSO, 0.1 M KCl, 50 mM MOPS–Tris (pH 7.0), and others as described in figure and table legends. The content of vanadate bound to the vesicles was determined according to the procedure of Goodno (1979) as described previously (Kubota et al., 1993).

Ca^{2+} Binding. The SR vesicles (10 mg/mL) were preincubated at 25 °C for 1 h in a medium containing 5 μM A23187, 0.2 mM EGTA, 0.1 M KCl, and 30 mM MOPS–Tris (pH 7.0), and the mixture was centrifuged. The pellet was washed with solution A by centrifugation and suspended in solution A. The vesicles (0.1 mg/mL) were then incubated at 25 °C for 30 s in a medium containing 20–199 μM $^{45}CaCl_2$, 0.15 mM EGTA, 5 mM $MgCl_2$, 0.1 M KCl, and 30 mM MOPS–Tris (pH 7.0), and the amount of bound $^{45}Ca^{2+}$ was determined by filtration on a membrane filter as described previously (Kubota et al., 1993). The content of bound $^{45}Ca^{2+}$ was obtained by subtracting the radioactivity on the filter through which the same solution without the vesicles was filtered.

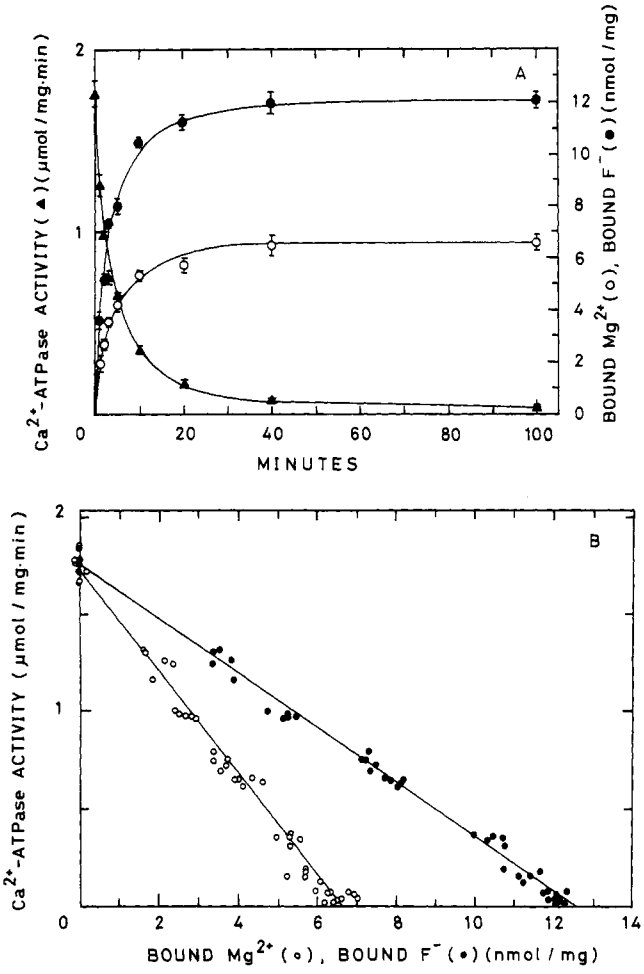

FIGURE 1: F^--induced inactivation of the Ca^{2+}-ATPase of SR vesicles and tight binding of Mg^{2+} and F^-. (A) The SR vesicles were preincubated as described under Experimental Procedures. The F^- treatment was started by addition of KF, and then the reaction was quenched at various times. At zero time, the reaction was quenched just before the addition of KF. The contents of tightly-bound Mg^{2+} and F^- as well as the Ca^{2+}-ATPase activity were determined. The contents of bound Mg^{2+} and F^- were plotted after subtraction of the contents of bound Mg^{2+} and F^- (0.88 ± 0.12 nmol of Mg^{2+}/mg and 0.17 ± 0.01 nmol of F^-/mg) at zero time. Vertical bars denote SD of the mean for five individual experiments. (B) The Ca^{2+}-ATPase activity was plotted vs the contents of bound Mg^{2+} and F^- after subtraction of the contents of bound Mg^{2+} and F^- at zero time in all the individual experiments in (A).

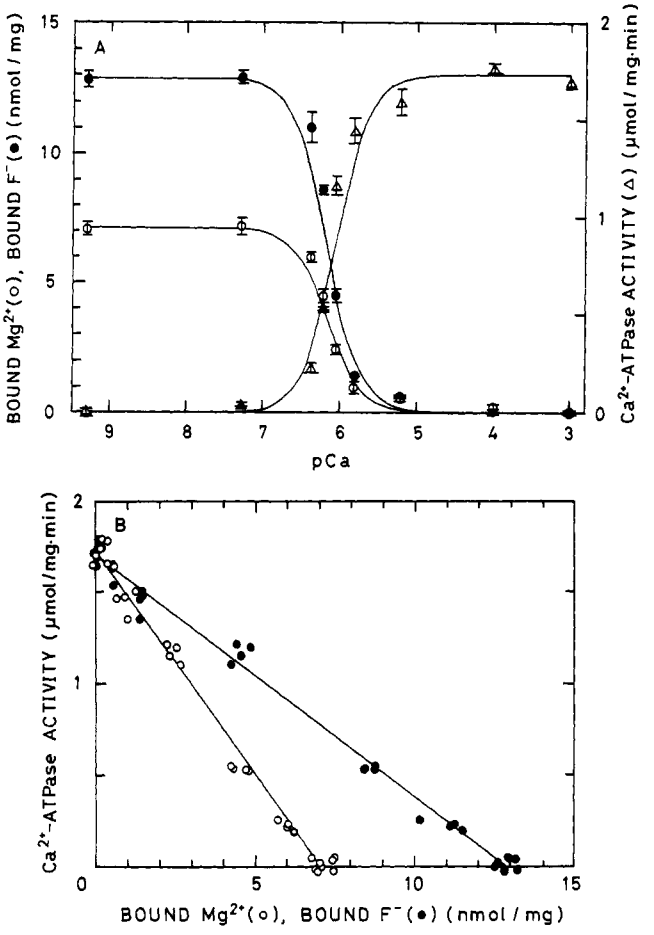

FIGURE 2: Protection of the Ca^{2+}-ATPase of SR vesicles by various concentrations of Ca^{2+} against the tight binding of Mg^{2+} and F^-. The SR vesicles were preincubated for 1 h as described under Experimental Procedures, and EGTA, $MgCl_2$, and $CaCl_2$ were added to give 1.02 mM EGTA, 8.36–8.98 mM $MgCl_2$, and 5.1 μM–2.04 mM $CaCl_2$. The mixture was further preincubated for 5 min, and the F^- treatment was started by addition of KF. The final composition of the reaction mixture was 0.66 mM KF (0.48 mM F^-), 8.2–8.8 mM $MgCl_2$ (8 mM Mg^{2+}), and 5 μM–2 mM $CaCl_2$, otherwise as described under Experimental Procedures. The reaction was quenched 1 h after the start. (A) The contents of tightly-bound Mg^{2+} and F^- as well as the Ca^{2+}-ATPase activity were determined. The Ca^{2+}-sensitive parts of the contents of bound Mg^{2+} and F^- were obtained by subtracting the contents of bound Mg^{2+} and F^- (1.15 ± 0.09 nmol of Mg^{2+}/mg and 0.40 ± 0.01 nmol of F^-/mg) at pCa 3 from the contents of bound Mg^{2+} and F^- at various pCa and were plotted vs pCa. Vertical bars denote SD of the mean for four individual experiments. (B) The Ca^{2+}-ATPase activity was plotted vs the Ca^{2+}-sensitive parts of the contents of bound Mg^{2+} and F^- in all the individual experiments in (A).

Others. [α-^{32}P]ATP and [γ-^{32}P]ATP were purchased from Amersham. Other materials were obtained as described previously (Kubota et al., 1993). Protein concentrations were determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard. Data analysis and calculation of the concentrations of free ligands and complexes were performed as described previously (Kubota et al., 1993).

RESULTS AND DISCUSSION

F^--Induced Inactivation of the Ca^{2+}-ATPase and Tight Binding of Mg^{2+} and F^- to the Ca^{2+}-ATPase of SR Vesicles. When the SR vesicles were treated with 0.65 mM F^- in the presence of 9 mM Mg^{2+}, the Ca^{2+}-ATPase activity decreased with time and almost completely disappeared in 40 min (Figure 1A). Coincidentally, Mg^{2+} and F^- did bind tightly to the vesicles. These results are consistent with the previous findings (obtained with the purified Ca^{2+}-ATPase) that the enzyme is inactivated by tight binding of Mg^{2+} and F^- to the catalytic site (Kubota et al., 1993). A plot of the Ca^{2+}-ATPase activity vs the content of tightly-bound Mg^{2+} or F^- gave a linear relationship (Figure 1B). The contents of tightly-bound ligands in the fully inactivated vesicles were found from the intercepts to be 6.65 nmol/mg for Mg^{2+} and 12.6 nmol/mg for F^-, being 2.0 and 3.8 times, respectively, the content of phosphorylation site (3.33 ± 0.06 nmol/mg; experiment 1 in Table I).

Protection of the Ca^{2+}-ATPase by Various Concentrations of Ca^{2+} against the Tight Binding of Mg^{2+} and F^-. The SR vesicles were completely protected by cooperative high-affinity Ca^{2+} binding against the tight binding of Mg^{2+} and F^- (Figure 2A). The Ca^{2+} concentration dependence of this protection corresponded to that of the protection against the F^--induced inactivation of the Ca^{2+}-ATPase. The dissociation constant (K_d) for Ca^{2+} and Hill coefficient were 0.67–0.86 μM and 2.0, respectively. A plot of the Ca^{2+}-ATPase activity vs the content of tightly-bound Mg^{2+} or F^- again gave a linear

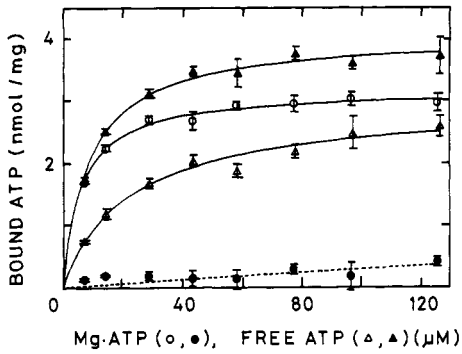

FIGURE 3: ATP binding to the Ca^{2+}-ATPase of F^--treated SR vesicles. The SR vesicles were treated with (●, ▲) or without (○, △) F^- for 100 min, otherwise as described for Figure 1. ATP binding to the vesicles was determined at various concentrations of [α-^{32}P]ATP, either in the presence of 20 mM $MgCl_2$ and 5 mM EGTA (○, ●) or in the absence of added $MgCl_2$ and presence of 5 mM EDTA (△, ▲). The content of bound [α-^{32}P]ATP was plotted vs the concentration of unbound Mg·[α-^{32}P]ATP (○, ●) or vs that of unbound metal-free [α-^{32}P]ATP (△, ▲). Vertical bars denote SD of the mean for five individual experiments. The solid lines represent least-squares fit of the data to the Michaelis equation in which the ligand concentrations indicated on the abscissa are used as concentrations of free substrate. The broken line represents least-squares fit of the data to the Michaelis equation in which the concentration of unbound metal-free ATP in the presence of added $MgCl_2$ is used as a concentration of free substrate.

relationship (Figure 2B). The contents of tightly-bound ligands in the fully inactivated vesicles obtained from the intercepts were 7.05 nmol/mg for Mg^{2+} and 12.9 nmol/mg for F^-, being 2.1 and 3.8 times, respectively, the content of phosphorylation site (3.43 ± 0.05 nmol/mg; experiment 2 in Table I).

ATP Binding to the Ca^{2+}-ATPase of F^--Treated SR Vesicles. ATP binding to the SR vesicles treated with or without F^- was determined at various concentrations of ATP in the absence of Ca^{2+}, and the data were analyzed by least-squares fitting to the Michaelis equation (Figure 3).

When 5 mM EDTA was present without added $MgCl_2$, the maximum extent of ATP binding to the F^--treated SR vesicles (obtained by extrapolation of the fitting curve) was 4.05 nmol/mg (see experiment 2 in Table I). This value is somewhat higher than the content of phosphorylation site (3.43 ± 0.05 nmol/mg; experiment 2 in Table I) obtained with the native preparation of SR vesicles used. It appears possible that this slight discrepancy is due to some errors inherent in extrapolation of the fitting curve. The K_d for metal-free ATP was 8.9 μM. This affinity was considerably higher than that for metal-free ATP (K_d = 22 μM) with the control vesicles (SR vesicles treated without F^-). These results show that metal-free ATP can bind to the catalytic site of the Ca^{2+}-ATPase in the F^--treated vesicles with a high affinity similar to the affinity for Mg·ATP of the catalytic site of the Ca^{2+}-ATPase in the control vesicles (K_d = 6.1 μM, see below). This high affinity for metal-free ATP in the F^--treated vesicles is likely due to an electrostatic attraction between the Mg^{2+} bound tightly to the catalytic site and the negatively-charged phosphate moiety of metal-free ATP. The observed low affinity for metal-free ATP with the control vesicles is consistent with the affinity for metal-free ATP with the native vesicles reported previously (Meissner, 1973).

When 20 mM $MgCl_2$ was present, the maximum level of ATP binding and K_d for Mg·ATP with the control vesicles were 3.18 nmol/mg and 6.1 μM, respectively. This maximum level of ATP binding is somewhat lower than the content of phosphorylation site (3.43 ± 0.05 nmol/mg; experiment 2 in Table I). This finding is consistent with the previous observations (Kubota et al., 1993) that the maximum extent of ATP binding to the purified Ca^{2+}-ATPase decreased to 80–85% of the content of the ATP-binding site when the Mg^{2+} concentration was raised to 20 mM. The high affinity for Mg·ATP with the control vesicles is in agreement with that for Mg·ATP with the native SR vesicles reported previously (Meissner, 1973; Dupont, 1977).

ATP binding in the presence of 20 mM $MgCl_2$ was almost entirely prevented by the F^- treatment. ATP binding remaining to a slight extent can be attributed to binding of metal-free ATP present at low concentrations (0.05–0.89 μM). In fact, the binding data (Figure 3, solid circles) are in fair agreement with the extent of binding of metal-free ATP (Figure 3, broken line) calculated from the concentrations of unbound metal-free ATP and the K_d for metal-free ATP (8.9 μM). These findings show that Mg·ATP cannot bind to the Ca^{2+}-ATPase of the F^--treated vesicles.

Lack of High-Affinity Vanadate Binding to the Ca^{2+}-ATPase of F^--Treated SR Vesicles. Vanadate has been reported to bind to phosphorylation site of the Ca^{2+}-ATPase as an analogue of P_i in the presence of Mg^{2+} (Dupont & Bennett, 1982). In this experiment, vanadate binding was determined at various concentrations of vanadate with or without Mg^{2+} in the presence of 40% (v/v) DMSO (Figure 4). When Mg^{2+} was absent, no significant vanadate binding occurred over the whole range of vanadate concentrations used either with the control vesicles or with the F^--treated vesicles. When Mg^{2+} was present, the extent of vanadate binding to the control vesicles increased with increasing concentration of vanadate and the binding was saturated with 1.7 μM vanadate. The low-affinity vanadate binding, which was found previously in the presence of 10% (v/v) DMSO (Yamasaki & Yamamoto, 1991), did not occur to a significant extent in the presence of 40% (v/v) DMSO. This allowed us to accurately determine the content of the high-affinity vanadate-binding site without interference from low-affinity

Table I: Stoichiometry of Tight Binding of Mg^{2+} and F^- to Phosphorylation and High-Affinity Binding of ATP, Vanadate, and Ca^{2+} [a]

	phosphorylation[b] (nmol/mg)	high-affinity binding			tight binding	
		ATP (nmol/mg)	vanadate[f] (nmol/mg)	Ca^{2+} [g] (nmol/mg)	Mg^{2+} (nmol/mg)	F^- (nmol/mg)
expt 1	3.33 ± 0.06 (n = 6)	3.54 ± 0.12[c] (n = 8) 3.59 ± 0.24[d] (n = 5)	3.34 ± 0.04 (n = 4)	6.98 ± 0.16 (n = 5)	6.65[h]	12.6[h]
expt 2	3.43 ± 0.05 (n = 6)	4.05[e]	3.50 ± 0.01 (n = 6)	7.03 ± 0.25 (n = 4)	7.05[i]	12.9[i]

[a] Experiments 1 and 2 were performed with different preparations of SR vesicles. [b] Determined as described under Experimental Procedures. [c] Determined at 79 μM [α-^{32}P]ATP in the presence of 5 mM $MgCl_2$ and 5 mM EGTA. [d] Determined at 98 μM [α-^{32}P]ATP, otherwise as above. [e] The maximum extent of ATP binding to the F^--treated SR vesicles in the presence of 5 mM EDTA and absence of added $MgCl_2$ in the experiment given in Figure 3, being obtained by extrapolation of the fitting curve. [f] Determined in the presence of 5 mM $MgCl_2$ at 1.5–3.0 μM vanadate in expt 1 or at 1.7–3.5 μM vanadate in expt 2. [g] Determined by subtracting the mean of the contents of bound Ca^{2+} in the F^--treated SR vesicles from that of the contents of bound Ca^{2+} in the control SR vesicles at 16.3 μM Ca^{2+}. [h] Obtained by extrapolation of the fitting line in Figure 1B. [i] Obtained by extrapolation of the fitting line in Figure 2B.

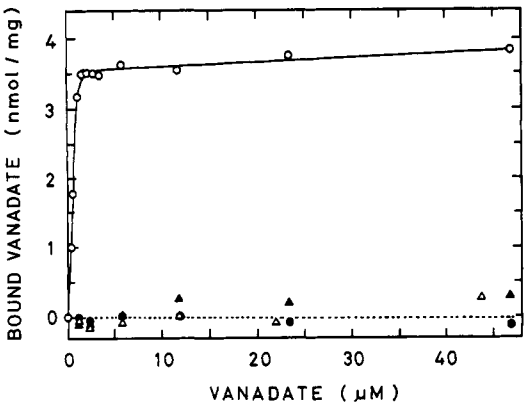

FIGURE 4: Lack of high-affinity vanadate binding to the Ca^{2+}-ATPase of F^--treated SR vesicles. The SR vesicles treated with F^- (F^--treated vesicles; ●, ▲) or without F^- (control vesicles; ○, △) were obtained as described for Figure 3. Vanadate binding to the vesicles was determined at various concentrations of vanadate, either in the presence of 5 mM $MgCl_2$ (○, ●) or in the absence of added $MgCl_2$ and presence of 5 mM EDTA (△, ▲). The content of bound vanadate was plotted vs the vanadate concentration.

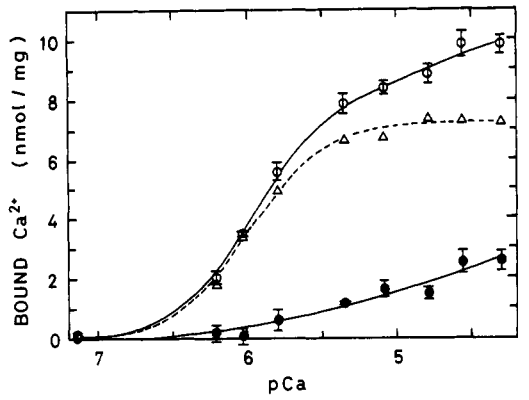

FIGURE 5: Lack of high-affinity Ca^{2+} binding to the Ca^{2+}-ATPase of F^--treated SR vesicles. The SR vesicles treated with F^- (F^--treated vesicles; ●) or without F^- (control vesicles; ○) were obtained as described for Figure 3. Ca^{2+} binding to the vesicles was determined at various concentrations of $^{45}Ca^{2+}$, and the content of bound $^{45}Ca^{2+}$ was plotted vs pCa. Vertical bars denote SD of the mean for four individual experiments. The difference between the contents of $^{45}Ca^{2+}$ bound to the F^--treated vesicles and those of $^{45}Ca^{2+}$ bound to the control vesicles is plotted (△).

vanadate binding. The content of the high-affinity vanadate-binding site thus determined was 3.50 ± 0.01 nmol/mg, being in good agreement with the content of phosphorylation site (3.43 ± 0.05 nmol/mg; experiment 2 in Table I). This vanadate binding was completely prevented by the F^- treatment. These findings indicate that vanadate cannot bind to phosphorylation site of the Ca^{2+}-ATPase in the F^--treated SR vesicles and further suggest that phosphorylation site is occupied by tightly-bound F^-.

Lack of High-Affinity Ca^{2+} Binding to the Ca^{2+}-ATPase of F^--Treated SR Vesicles. Ca^{2+} binding to the SR vesicles treated with or without F^- was determined at various concentrations of Ca^{2+} (Figure 5). The content of the high-affinity Ca^{2+}-binding site in the control vesicles was 7.03 ± 0.25 nmol/mg (broken line in Figure 5 and experiment 2 in Table I), being twice the content of phosphorylation site (3.43 ± 0.05 nmol/mg; experiment 2 in Table I). The K_d and Hill coefficient were 1.1 μM and 1.8, respectively. This cooperative high-affinity Ca^{2+} binding was nearly completely prevented by the F^- treatment. This finding is consistent with the results reported previously (Murphy & Coll, 1992b; Kubota et al., 1993).

Stoichiometry between the Contents of Tightly-Bound Mg^{2+} and F^- in the Completely Inactivated SR Vesicles and the Contents of Phosphorylation Site and High-Affinity ATP-, Vanadate-, and Ca^{2+}-Binding Sites. The observed content of the high-affinity ATP- or vanadate-binding site was in excellent agreement with that of phosphorylation site. In addition, the observed content of the high-affinity Ca^{2+}-binding site was just twice that of phosphorylation site (Table I). This stoichiometry among the high-affinity ATP- or vanadate-binding sites, the phosphorylation site, and the high-affinity Ca^{2+}-binding site is in substantial accord with that obtained previously in other laboratories (Meissner, 1973; Dupont et al., 1982; Andersen & Møller, 1985; Yamasaki & Yamamoto, 1991). This suggests that the present determination of the contents of these sites is reliable.

The linear relationship existing between the extent of the enzyme inactivation and the contents of tightly-bound Mg^{2+} and F^- (Figure 1B) gives strong support to the conclusion that the enzyme inactivation is due to tight binding of these ligands. This causal relation is further manifested by the finding that the Ca^{2+}-induced protection against the enzyme inactivation is closely in parallel with the Ca^{2+}-induced protection against the tight ligand binding (Figure 2). The contents of tightly-bound Mg^{2+} and F^- in the completely inactivated SR vesicles were 2 and 4 times, respectively, the content of phosphorylation site or that of high-affinity ATP- or vanadate-binding site (Table I). These results demonstrate that tight binding of 2 mol of Mg^{2+} and 4 mol of F^- per mole of phosphorylation site, per mole of high-affinity ATP- or vanadate-binding site, and per 2 mol of high-affinity Ca^{2+}-binding site leads to a complete inactivation of the Ca^{2+}-ATPase in SR vesicles. This stoichiometry contrasts sharply with that suggested previously by Coll and Murphy (1992).

Two models for the observed stoichiometry are tenable: (1) All Ca^{2+}-ATPase molecules present are catalytically active (i.e., these ATPase molecules can be phosphorylated and can bind ATP, vanadate, and Ca^{2+} with high affinities), and two Mg^{2+} and four F^- bind to each Ca^{2+}-ATPase molecule. (2) Half of the Ca^{2+}-ATPase molecules present are catalytically active, the other half being catalytically inactive (i.e., these ATPase molecules cannot be phosphorylated and cannot bind ATP, vanadate, and Ca^{2+} with high affinities), and one Mg^{2+} and two F^- bind equally to each of the catalytically active and inactive Ca^{2+}-ATPase molecules. The latter model is based on the previous kinetic observations (Kubota et al., 1993) that the reciprocal of the first-order rate constant of the F^--induced enzyme inactivation is proportional to the reciprocal of the Mg^{2+} concentration and to the reciprocal of the square of the F^- concentration. According to this model, an intermolecular ATPase–ATPase interaction should be induced by high-affinity Ca^{2+} binding because the tight ligand binding to all the ATPase molecules is entirely prevented by the Ca^{2+} binding to the catalytically active ATPase molecules alone.

Recently, Troullier et al. (1992) have shown that 4.8 nmol of AlF_4^-/mg of protein binds to the SR Ca^{2+}-ATPase by a quasi-irreversible process to form an abortive complex. This would fit with one AlF_4^- per phosphorylation site since they have obtained the content of phosphorylation site equivalent to 3–4 nmol/mg of protein in their preparation of SR vesicles. Their data show that AlF_4^- is an inhibitory analogue of P_i very similar to vanadate, another well-known analogue of P_i. It is, therefore, conceivable that the stoichiometry of AlF_4^- binding to phosphorylation in their experiments is in agreement with that of vanadate binding (but not F^- binding) to phosphorylation in our experiments.

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