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Functional Characterization of Lanthanide Binding Sites in the Sarcoplasmic Reticulum Ca^{2+}-ATPase: Do Lanthanide Ions Bind to the Calcium Transport Site?†

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ABSTRACT: Gd^{3+} binding sites on the purified Ca^{2+}-ATPase of sarcoplasmic reticulum were characterized at 2 and 6 °C and pH 7.0 under conditions in which $^{45}Ca^{2+}$ and $^{54}Mn^{2+}$ specifically labeled the calcium transport site and the catalytic site of the enzyme, respectively. We detected several classes of Gd^{3+} binding sites that affected enzyme function: (a) Gd^{3+} exchanged with $^{54}Mn^{2+}$ of the ^{54}MnATP complex bound at the catalytic site. This permitted slow phosphorylation of the enzyme when two Ca^{2+} ions were bound at the transport site. The Gd^{3+} ion bound at the catalytic site inhibited decomposition of the ADP-sensitive phosphoenzyme. (b) High-affinity binding of Gd^{3+} to site(s) distinct from both the transport site and the catalytic site inhibited the decomposition of the ADP-sensitive phosphoenzyme. (c) Gd^{3+} enhanced 4-nitro-2,1,3-benzoxadiazole (NBD) fluorescence in NBD-modified enzyme by probably binding to the Mg^{2+} site that is distinct from both the transport site and the catalytic site. (d) Gd^{3+} inhibited high-affinity binding of $^{45}Ca^{2+}$ to the transport site not by directly competing with Ca^{2+} for the transport site but by occupying site(s) other than the transport site. This conclusion was based mainly on the result of kinetic analysis of displacement of the enzyme-bound $^{45}Ca^{2+}$ ions by Gd^{3+} and vice versa, and the inability of Gd^{3+} to phosphorylate the enzyme under conditions in which GdATP served as a substrate. These results strongly suggest that Ln^{3+} ions cannot be used as probes to structurally and functionally characterize the calcium transport site on the Ca^{2+}-ATPase.

The Ca^{2+}-ATPase of the sarcoplasmic reticulum (SR)[1] utilizes the magnesium-ATP complex as a physiological substrate to drive active transport of Ca^{2+} across the SR membrane (Vianna, 1975; Martonosi & Beeler, 1983). For the rapid turnover of the ATPase, high-affinity binding of 3 mol of divalent cations is minimally required, of which 2 mol is Ca^{2+} ions bound at the calcium-specific transport site while the remainder is 1 mol bound at the catalytic site as a component of the divalent cation-ATP complex (Shigekawa et al., 1983b; Ogurusu et al., 1991). The divalent cation at the catalytic ATP site remains bound until the phosphoenzyme intermediate is hydrolyzed. This divalent cation presumably determines the catalytic rate of each reaction step of ATP hydrolysis (Shigekawa et al., 1983b; Ogurusu et al., 1991). In addition to these high-affinity sites, several classes of low-affinity sites for divalent cations have been reported for the Ca^{2+}-ATPase (Ikemoto, 1974; Kalbitzer et al., 1978; Guillain et al., 1982; Loomis et al., 1982; Champeil et al., 1983; Highsmith & Head, 1983; Wakabayashi et al., 1986, 1987, 1990b), although the functional roles of some of these sites remain unclear.

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[1] Abbreviations: SR, sarcoplasmic reticulum; Ln^{3+}, lanthanide ion(s); Mops, 3-(*N*-morpholino)propanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; NBD-Cl, 7-chloro-4-nitro-2,1,3-benzoxadiazole.

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Trivalent lanthanide ions (Ln^{3+}) have been shown to inhibit ATPase activity and Ca^{2+} binding to the transport site of Ca^{2+}-ATPase (Chevallier & Butow, 1971; Krasnow, 1972; Meissner, 1973; Chiesi & Inesi, 1979; Highsmith & Head, 1983; Itoh & Kawakita, 1984; Girardet et al., 1989; Fujimori & Jenks, 1990; Squier et al., 1990). Thus, the ATPase inhibition was considered to be due to binding of Ln^{3+} ions to the transport site of the enzyme (Stephans & Grisham, 1979; Highsmith & Head, 1983; Scott, 1984; Squier et al., 1990). Ln^{3+} ions are unique in that they have ionic radii, coordination geometry, and donor atom preference very similar to those of Ca^{2+} and that some of them have useful spectroscopic and magnetic properties (Evans, 1989). In the case of SR Ca^{2+}-ATPase, the latter properties have been utilized to study the physical state of the bound Ln^{3+} ions as well as to estimate the distances between the transport site and the other functional site of the enzyme (Stephans & Grisham, 1979; Highsmith & Murphy, 1984; Scott, 1985; Herrman et al., 1986).

However, direct comparison of the effective concentrations of Gd^{3+} for the displacement of radioactive calcium bound at the transport site and for the inhibition of ATPase activity showed that Gd^{3+} bound to the site(s) other than the transport sites inhibits the enzyme activity (Itoh & Kawakita, 1984). A recent kinetic study of the effect of La^{3+} has suggested that the inhibition of enzyme activity is caused by binding of La^{3+} to the catalytic ATP site (Fujimori & Jencks, 1990). In addition to these results, fluorescence measurements have yielded results showing the presence of several classes of binding sites for Tb^{3+} on the enzyme (Highsmith & Head, 1983; Girardet et al., 1989). Thus, Ln^{3+} ions probably can bind to many divalent cation binding sites on the Ca^{2+}-ATPase.

Recently, we (Ogurusu et al., 1991) have successfully established the experimental conditions under which the transport site and catalytic site were specifically labeled with radioactive Ca^{2+} and Mn^{2+}, respectively. In the present study, by employing these conditions and by following the fates of these radioactive labels, we characterized the interactions of Gd^{3+} with binding sites on the Ca^{2+}-ATPase. We presented evidence that although Gd^{3+} is able to inhibit Ca^{2+}-ATPase by binding to several classes of binding sites on the enzyme, it does not bind to the calcium-specific transport site directly with high affinity.

Materials and Methods

Sarcoplasmic reticulum vesicles and the purified ATPase protein were prepared from rabbit white skeletal muscle as described previously (Shigekawa et al., 1983a). The modification of the enzyme with 7-chloro-4-nitro-2,1,3-benzoxadiazole (NBD-Cl) was performed also as described (Wakabayashi et al., 1990a). One milligram of the ATPase protein was contaminated with 10–20 nmol of calcium, as estimated by atomic absorption spectrometry. The contaminating calcium was taken into account for calculation of the total calcium participating in the reaction. For some experiments (Table I, Figure 4 inset, and Figures 6 and 7), the contaminating calcium was removed from the ATPase protein by treatment with EGTA; the ATPase protein (5 mg/mL) was incubated for 2 min at 0 °C in 1 M KCl, 0.5 M sucrose, 50 mM Mes/Tris (pH 6.5), and 5 mM EGTA and then washed 3 times by centrifugation (400000g for 6 min) with a solution containing 1 M KCl, 0.5 M sucrose, and 50 mM Mes/Tris (pH 6.5).

The ATPase reaction was started by the addition of a mixture of ATP, $MnCl_2$, and $GdCl_3$ to the reaction medium. Unless otherwise stated, the ATPase reaction was carried out at 2 °C in a medium containing 0.3 mg/mL ATPase protein, 30 mM Mops/KOH (pH 7.0), 0.3 M KCl, 30 or 50 μM ATP or [γ-^{32}P]ATP, 0 or 20 μM $CaCl_2$ or $^{45}CaCl_2$, 0–100 μM $MnCl_2$ or $^{54}MnCl_2$, and 0–400 μM $GdCl_3$.

Levels of $^{45}Ca^{2+}$, $^{54}Mn^{2+}$, and ^{32}P label bound to the enzyme were measured by using the Biologic filtration apparatus (Dupont, 1984) 15 or 60 s after the start of enzyme phosphorylation. Time courses for release of the enzyme-bound radioactive labels (^{45}Ca, ^{54}Mn, and ^{32}P) and for binding of $^{45}Ca^{2+}$ to the enzyme were measured by using the same filtration apparatus. In the latter experiments, the enzyme was first immobilized on a 0.65-μm Millipore filter (DAWP) by suction and then washed for a predetermined period of time with various washing media (Wakabayashi et al., 1986). In some experiments (Figures 6 and 7), 10 mM [^{3}H]glucose was included in the washing medium to determine the filter wet volume for calculation of unbound $^{45}Ca^{2+}$.

ATPase activity and levels of the total acid-stable phosphoenzyme were measured as described previously (Shigekawa et al., 1983a). The time course of rapid phosphorylation was followed by using a simple mixing apparatus as described previously (Kanazawa et al., 1970). Fluorescence measurements were performed also as described (Wakabayashi et al., 1990a). Protein concentration was determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard.

Equilibrium measurements of intrinsic protein fluorescence and NBD fluorescence were carried out on a Hitachi MPF 4 spectrofluorometer with excitation at 290 nm and emission at 330 nm for intrinsic protein fluorescence and with excitation at 430 nm and emission at 510 nm for NBD fluorescence.

The concentrations of ionized lanthanide ions were not calculated in this study. The total concentrations of these ions are indicated in the present paper.

$^{45}CaCl_2$ and [^{3}H]glucose were purchased from New England Nuclear. $^{54}MnCl_2$ was purchased from Amersham. Pyruvate kinase (type III) and phosphoenolpyruvate (monopotassium salt) were purchased from Sigma. Tris/ATP and [γ-^{32}P]ATP were prepared as described previously (Shigekawa et al., 1983a). $GdCl_3 \cdot 6H_2O$ (99.9% pure) and $LaCl_3 \cdot 7H_2O$ (99.9% pure) were purchased from Wako Pure Chemical and dissolved in water just before use.

Results

Interaction of Gd^{3+} with the Catalytic Site of Ca^{2+}-ATPase. In our previous study (Ogurusu et al., 1991), we established the conditions under which the transport site and the catalytic site of Ca^{2+}-ATPase can be labeled with $^{45}Ca^{2+}$ and $^{54}Mn^{2+}$, respectively, during the ATPase reaction. Employing these conditions, we studied the effect of Gd^{3+} on the fates of the $^{45}Ca^{2+}$ and $^{54}Mn^{2+}$ ions bound to the enzyme (Figure 1). In the same experiment, we also studied the effect of Gd^{3+} on the levels of the acid-stable phosphoenzyme intermediate and of the enzyme-bound ^{32}P label derived from [γ-^{32}P]ATP.

In these experiments, the ATPase reaction was started by the addition of a mixture of ATP, Mn^{2+}, and Gd^{3+} (final concentrations, 30, 60, and 0–400 μM, respectively) to the enzyme preincubated with 20 μM Ca^{2+}. Figure 1 shows that the levels of both enzyme-bound $^{54}Mn^{2+}$ (Δ) and P_i liberation (*) measured 1 min after the start of ATPase reaction decreased markedly with increasing Gd^{3+} concentrations and became negligible at Gd^{3+} concentrations above 50 μM. Interestingly, inhibition of P_i liberation occurred apparently at slightly lower concentrations of Gd^{3+} than that of $^{54}Mn^{2+}$ binding (Figure 1).

On the other hand, the level of acid-stable phosphoenzyme measured also at 1 min after the start of ATPase reaction was

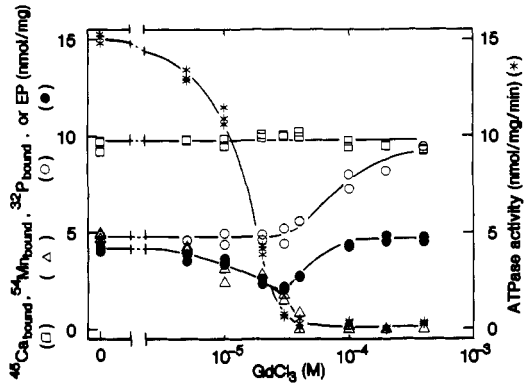


FIGURE 1: Dependence on Gd^{3+} concentration of the ATPase activity and the acid-stable phosphoenzyme level and of the levels of $^{45}Ca^{2+}$, $^{54}Mn^{2+}$, and ^{32}P label bound to the Ca^{2+}-ATPase. The ATPase reaction was started by the addition of a mixture of $MnCl_2$ or $^{54}MnCl_2$, ATP or [γ-^{32}P]ATP, and $GdCl_3$ (final concentrations, 60, 30 and 0–400 μM, respectively) to the ATPase protein (0.3 mg/mL) which had been preincubated at 2 °C with either 20 μM $^{40}CaCl_2$ or 20 μM $^{45}CaCl_2$ in 30 mM Mops/KOH (pH 7.0) and 0.3 M KCl. Sixty seconds later, the amount of P_i liberated and levels of phosphoenzyme and bound radioactive labels were determined as described under Materials and Methods.

found to be a complicated function of the Gd^{3+} concentration (Figure 1, ●); it decreased with increasing Gd^{3+} concentrations up to 30 μM and then increased at higher Gd^{3+} concentrations, reaching the maximum which was equal to the maximum level (5.0 ± 0.2 nmol/mg, n = 5) obtained with MgATP in the absence of Gd^{3+}. In the absence of Gd^{3+}, the level of the acid-stable phosphoenzyme was almost identical with that of ^{32}P label bound to the enzyme (Figure 1, ○). However, the latter exceeded the former as the Gd^{3+} concentration became higher and reached about 9 nmol/mg at 400 μM Gd^{3+}. This net increase of the latter relative to the former was clearly due to increased binding of [γ-^{32}P]ATP to the enzyme. A high level of nucleotide binding was also observed by Girardet et al. (1989) in the presence of Tb^{3+}.

In contrast, the level of the enzyme-bound $^{45}Ca^{2+}$ (9–10 nmol/mg) did not change when the Gd^{3+} concentration was increased from 0 to 400 μM (□). $^{45}Ca^{2+}$ ions were bound almost exclusively at the transport site on the phosphoenzyme. This is because only a negligible amount of $^{45}Ca^{2+}$ (0.1–0.6 nmol/mg) was exchanged with the enzyme-bound nonradioactive calcium, when enzyme phosphorylation was performed as in Figure 1 except that it was started by the addition of a mixture of Gd^{3+}, ATP, Mn^{2+}, and a trace amount of radioactive Ca^{2+} to the enzyme which had been preincubated with nonradioactive Ca^{2+}. If nonradioactive calcium was bound at the catalytic site of phosphoenzyme, calcium isotope exchange would have occurred, because the calcium bound at the catalytic site is exchangeable (Wakabayashi & Shigekawa, 1984).

Our previous data (Shigekawa et al., 1983b; Ogurusu et al., 1991) indicated that binding of at least 3 mol of divalent cations, of which 2 mol is Ca^{2+} ions bound to the transport site, is required for formation of the phosphoenzyme. The results of Figure 1 clearly show that Gd^{3+} replaced Mn^{2+} bound at the catalytic site on the phosphoenzyme as the Gd^{3+} concentration increased in the reaction medium. Thus, they provide direct evidence that GdATP serves as a substrate for phosphorylation of the Ca^{2+}-ATPase. In the presence of 200 μM Gd^{3+}, where no $^{54}Mn^{2+}$ binding was observed (Figure 1), the initial rate of enzyme phosphorylation by GdATP was 2.0 nmol mg^{-1} s^{-1}, which was about 5 times slower than that by MnATP in the absence of Gd^{3+}. This result is consistent with a recent report by Hanel and Jencks (1990), who observed slow

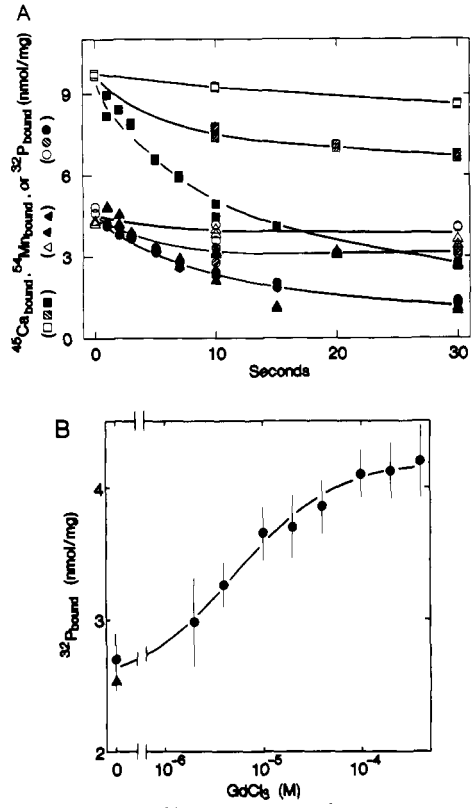


FIGURE 2: Effect of Gd^{3+} concentration on the release of $^{45}Ca^{2+}$, $^{54}Mn^{2+}$, and ^{32}P label from the ADP-sensitive phosphoenzyme formed from MnATP. (A) The ATPase protein (0.3 mg/mL) was phosphorylated for 15 s at 2 °C with 50 μM ATP or [γ-^{32}P]ATP in 100 μM $MnCl_2$ or $^{54}MnCl_2$, 20 μM $CaCl_2$ or $^{45}CaCl_2$, 1 mM phosphoenolpyruvate, and 0.15 mg/mL pyruvate kinase. The phosphoenzyme was then immobilized on the filter and washed at 2 °C with media containing 30 mM Mops/KOH (pH 7.0), 0.3 M KCl, and either 1 mM EGTA (solid symbols), 20 μM $GdCl_3$ (hatched symbols), or 1 mM $GdCl_3$ (open symbols) as described under Materials and Methods. (B) The ATPase was phosphorylated as in (A). The phosphoenzyme was washed as in (A) for 10 s at 2 °C with medium containing 1 mM EGTA (▲) or 0–400 μM $GdCl_3$ (●). The amount of the remaining bound ^{32}P label was plotted as a function of Gd^{3+} concentration. All determinations were performed in triplicate. Each vertical bar shows the size of the standard deviation.

phosphorylation of the Ca^{2+}-ATPase by LaATP.

Effect of Gd^{3+} on Decomposition of Phosphoenzyme. An intriguing question is how Gd^{3+} inhibits ATPase activity. Under the conditions of Figure 1, inhibition of Ca^{2+} binding to the transport site by Gd^{3+} was unlikely, because the level of the enzyme-bound Ca^{2+} did not change (Figure 1). On the other hand, decomposition of the phosphoenzyme formed in the presence of 100–400 μM Gd^{3+} was extremely slow, because ATPase activity was negligible in spite of the maximum level of the phosphoenzyme (Figure 1).

To elucidate the mechanism for inhibition of the phosphoenzyme decomposition by Gd^{3+}, we examined the effect of added Gd^{3+} on decomposition of the ADP-sensitive phosphoenzyme formed from MnATP (Figure 2). As shown in Figure 2A, Gd^{3+} added to the washing medium inhibited release of $^{45}Ca^{2+}$, $^{54}Mn^{2+}$, and ^{32}P label bound to the phosphoenzyme in a concentration-dependent manner. During the phosphoenzyme decomposition, the stoichiometric ratio between the amounts of enzyme-bound $^{45}Ca^{2+}$, $^{54}Mn^{2+}$, and ^{32}P label was mostly maintained at 2, 1, and 1 in the presence of 0, 20, or 1000 μM Gd^{3+}, indicating that the transport site and the catalytic site of the phosphoenzyme remained occupied by Ca^{2+} and Mn^{2+}, respectively. Figure 2B shows the Gd^{3+} concentration dependence of the level of ^{32}P label which re-

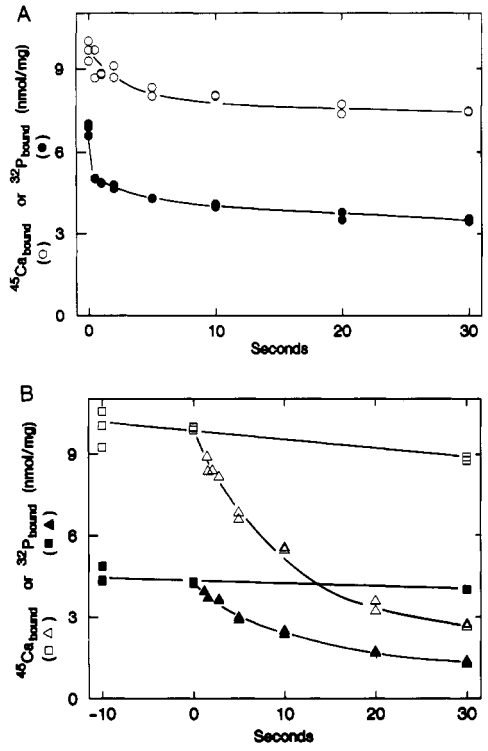


FIGURE 3: Decomposition of the ADP-sensitive phosphoenzyme formed from either MnATP or GdATP. (A) Enzyme phosphorylation was started by the addition of a mixture of $GdCl_3$ and ATP or [γ-^{32}P]ATP (final concentrations 100 and 30 μM, respectively) to the ATPase protein (0.3 mg/mL) which had been preincubated at 2 °C with either 20 μM $CaCl_2$ or $^{45}CaCl_2$ in 30 mM Mops/KOH (pH 7.0) and 0.3 M KCl. Sixty seconds later, the phosphoenzyme was washed as described under Materials and Methods with 0.3 M KCl, 30 mM Mops/KOH (pH 7.0), and 1 mM EGTA. (B) The enzyme was phosphorylated with MnATP as described in the legend to Figure 2. Fifteen seconds later, the phosphoenzyme was washed with a medium containing 1 mM $GdCl_3$ to inhibit decomposition of the phosphoenzyme. Ten seconds later (zero time in the figure), the phosphoenzyme was washed with 0.3 M KCl, 30 mM Mops/KOH (pH 7.0), and 1 mM $GdCl_3$ (□, ■) or 1 mM EGTA (△, ▲) for the periods of time indicated in the figure.

mained bound to the enzyme after the 10-s washing with the Gd^{3+}-containing media. The figure shows that 50% inhibition of phosphoenzyme decomposition was observed at about 10 μM Gd^{3+}. These results indicate that low concentrations of Gd^{3+} inhibit decomposition of the ADP-sensitive phosphoenzyme by binding to site(s) other than the Ca^{2+} transport site and the catalytic site.

Next, we examined the effect of Gd^{3+} bound at the catalytic site on decomposition of the ADP-sensitive phosphoenzyme. As shown in Figure 3A, release of $^{45}Ca^{2+}$ and ^{32}P label from the phosphoenzyme formed from GdATP was very slow even in the presence of excess EGTA, although the portion of bound ^{32}P label exceeding the phosphoenzyme level (cf. Figure 1) was released rapidly within 1 s. During this washing with EGTA, the stoichiometry of binding of Ca^{2+} to the phosphoenzyme was maintained at about 2. Thus, the catalytic site on the phosphoenzyme remained occupied by Gd^{3+}. This is because we showed previously that removal of metal from the catalytic site on the ADP-sensitive phosphoenzyme induces loss of Ca^{2+} from the transport site, thus decreasing the value for the Ca^{2+}/P label stoichiometry (Wakabayashi & Shigekawa, 1984; Wakabayashi et al., 1987).

In Figure 3B, we performed a control experiment for the experiment shown in Figure 3A. In this experiment, the ADP-sensitive phosphoenzyme was formed from MnATP, and its decomposition was inhibited by the addition of 1 mM Gd^{3+}

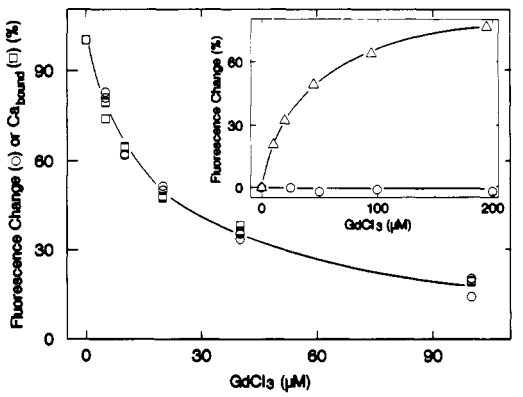


FIGURE 4: Effect of Gd^{3+} concentration on the levels of Ca^{2+} binding and the intrinsic fluorescence of the Ca^{2+}-ATPase. Equilibrium levels of $^{45}Ca^{2+}$ bound and the intrinsic fluorescence were measured at 6 °C in the reaction medium containing 30 mM Mops/KOH (pH 7.0), 0.3 M KCl, 2 mM $MgCl_2$, 20 μM $CaCl_2$ or $^{45}CaCl_2$, 0–200 μM $GdCl_3$, and 0.1 mg/mL ATPase protein. In the inset, the equilibrium levels of the intrinsic protein fluorescence in the unmodified enzyme (○) or NBD fluorescence in the NBD-modified enzyme (△) were measured under the same experimental conditions except that $CaCl_2$ was not added to the reaction medium. The levels of calcium bound and fluorescence obtained in the presence of 20 μM $CaCl_2$ were taken as 100%.

(cf. Figure 2). Subsequent addition of excess EGTA was able to abolish the Gd^{3+}-induced inhibition of the phosphoenzyme decomposition, indicating that the inhibitory Gd^{3+} bound at site(s) other than the transport site and the catalytic site on the phosphoenzyme was readily removed by added EGTA. From the results shown in Figure 3A,B, we concluded that Gd^{3+} bound at the catalytic site also inhibits the decomposition of the ADP-sensitive phosphoenzyme.

Interaction of Gd^{3+} with the Transport Site of Ca^{2+}-ATPase. It is well-known that binding of Ca^{2+} to the transport site of Ca^{2+}-ATPase enhances intrinsic tryptophan fluorescence (Dupont, 1976, 1982; Champeil et al., 1983). Addition of Gd^{3+} to the reaction medium containing 0.1 mg/mL Ca^{2+}-ATPase, 20 μM Ca^{2+} or $^{45}Ca^{2+}$, and 2 mM Mg^{2+} resulted in parallel inhibition of both $^{45}Ca^{2+}$ binding and intrinsic fluorescence enhancement, which occurred with the same $K_{1/2}$ values of 20 μM for Gd^{3+} (Figure 4). A $K_{1/2}$ value of 20 μM was also obtained when inhibition of $^{45}Ca^{2+}$ binding by Gd^{3+} was studied with 0.3 mg/mL Ca^{2+}-ATPase, a concentration of the enzyme which was used in all the other experiments of this study. On the other hand, Gd^{3+} was found not to be able to enhance the intrinsic tryptophan fluorescence in the absence of added Ca^{2+} (Figure 4, inset). Gd^{3+}, however, induced a rise of NBD fluorescence in the NBD-modified enzyme (Wakabayashi et al., 1990a) in the absence of added Ca^{2+} (Figure 4, inset). The inhibitions of Ca^{2+} binding and intrinsic fluorescence enhancement by other Ln^{3+} ions have also been reported by other workers (Girardet al al., 1989; Squier et al., 1990).

Next, we examined the effect of La^{3+} or Gd^{3+} on ATP-dependent phosphorylation of the Ca^{2+}-ATPase. In the absence of added Ca^{2+} and added Ln^{3+} ions, we detected the formation of a low level of phosphoenzyme (Table I), which was presumably due to the Ca^{2+} ions contaminating the reaction medium. Addition of Gd^{3+} or La^{3+} did not allow new formation of phosphoenzyme in the absence of added Ca^{2+}. However, addition of a low concentration of Ca^{2+} (20 μM) to the same Ln^{3+}-containing medium resulted in formation of high levels of phosphoenzyme (Table I). Thus, Ln^{3+} ions could not replace Ca^{2+} in the activation of enzyme phosphorylation. These results contradict recent data obtained by Squier et al.

Table I: Effect of the Ln^{3+} Ion on ATP-Dependent Phosphorylation of Ca^{2+}-ATPase[a]

$[Ln^{3+}]$ (μM)	phosphoenzyme level (nmol/mg)	
	no added $CaCl_2$	plus 20 μM $CaCl_2$
no addition	0.6 ± 0.1	4.9 ± 0.1
$GdCl_3$		
50	0.3 ± 0.0	
100	0.0 ± 0.0	3.0 ± 0.2
200	0.0 ± 0.0	1.7 ± 0.3
$LaCl_3$		
50	0.3 ± 0.1	
100	0.0 ± 0.0	3.2 ± 0.1
200	0.0 ± 0.0	1.7 ± 0.1

[a] The acid-stable phosphoenzyme level was measured 1 min after the addition of [γ-^{32}P]ATP (final concentration, 30 μM) to the reaction medium containing 0.3 mg/mL ATPase protein, 30 mM Mops/KOH (pH 7.0), 0.3 M KCl, 0 or 20 μM $CaCl_2$, and the indicated concentrations of La^{3+} or Gd^{3+}. The values shown in the table represent the mean ± SD of triplicate determinations.

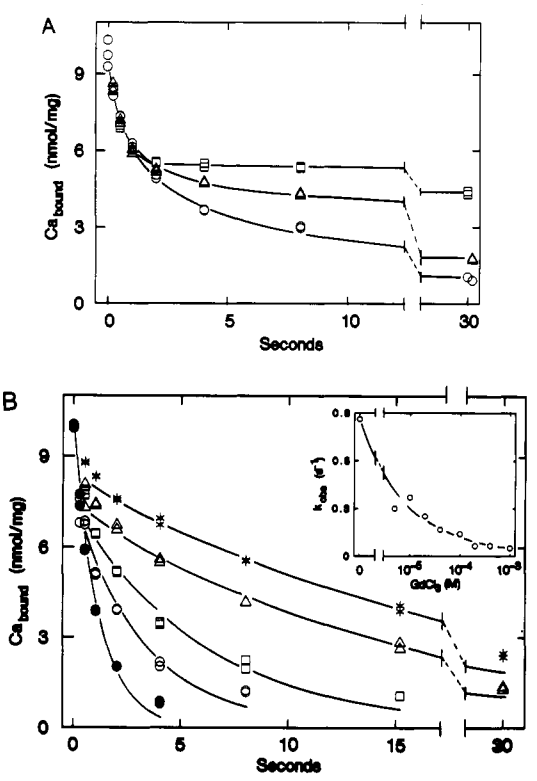


FIGURE 5: Release of bound $^{45}Ca^{2+}$ in the presence of unlabeled Ca^{2+} (A) or Gd^{3+} (B). The ATPase protein (0.3 mg/mL) was preincubated at 6 °C with 20 μM $^{45}CaCl_2$, 0.3 M KCl, 2 mM $MgCl_2$, and 30 mM Mops/KOH (pH 7.0). Then the enzyme was immobilized on the filter and washed at 6 °C with a medium containing 30 mM Mops/KOH (pH 7.0), 0.3 M KCl, 2 mM $MgCl_2$, and the following addition: in (A), 20 (○), 100 (△), or 1000 μM (□) $^{40}CaCl_2$; in (B), 1 mM EGTA (●) or 5 (○), 40 (□), 200 (△), or 1000 μM (*) $GdCl_3$. In (B), lines are drawn for first-order rate constants of 0.86 (●), 0.30 (○), 0.17 (□), 0.06 (△), and 0.05 s^{-1} (*). The inset in (B) shows the Gd^{3+} concentration dependence of the observed "off" rate constant (see text).

(1990) but are consistent with the result reported by Itoh and Kawakita (1984). Since GdATP can serve as a substrate for enzyme phosphorylation (see above), our data show that Ln^{3+} ions cannot function as an analogue for Ca^{2+} at the transport site.

Inabilities of Gd^{3+} to enhance the intrinsic fluorescence (Figure 4, inset) and to induce formation of the phosphoenzyme (Table I) in the Ca^{2+}-free enzyme question the validity of the widely accepted view that Ln^{3+} ions bind to the transport site directly. In the experiments shown below, we studied the effects of Gd^{3+} on the kinetics of binding to and release of Ca^{2+}

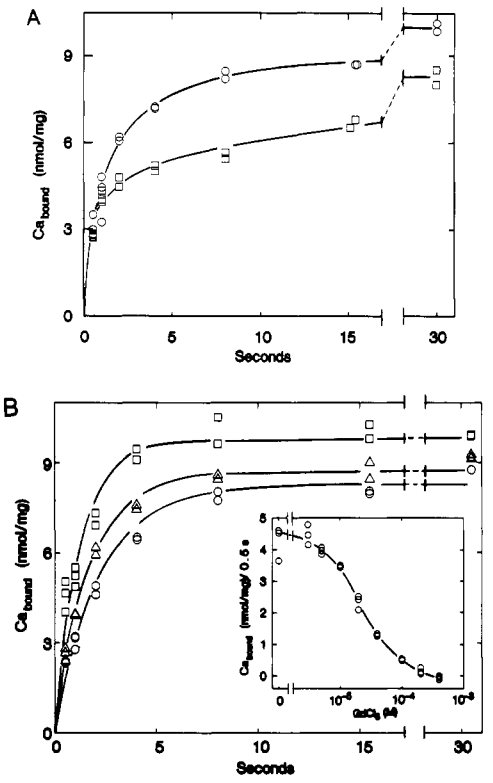


FIGURE 6: Binding of $^{45}Ca^{2+}$ to the ATPase which had been equilibrated with unlabeled Ca^{2+} or Gd^{3+}. The ATPase protein (0.3 mg/mL) was preequilibrated at 6 °C with either 30 μM $^{40}CaCl_2$ (A) or 30 μM $GdCl_3$ (B), 30 mM Mops/KOH (pH 7.0), 0.3 M KCl, and 2 mM $MgCl_2$. Then the enzyme was washed at 6 °C with a medium containing 30 mM Mops/KOH (pH 7.0), 0.3 M KCl, 2 mM $MgCl_2$, 10 mM [^{3}H]glucose, and 20 (○), 40 (△), or 100 μM (□) $^{45}CaCl_2$ by the procedure described under Materials and Methods. In (B) the lines are drawn to fit first-order rate constants of 0.47 (○), 0.58 (△), and 0.82 s^{-1} (□). In the inset in (B), the enzyme preincubated with the indicated concentrations of Gd^{3+} was washed for 0.5 s with a medium containing 30 mM Mops/KOH (pH 7.0), 0.3 M KCl, 2 mM $MgCl_2$, and 20 μM $^{45}CaCl_2$, and the amount of $^{45}Ca^{2+}$ bound to the enzyme was plotted as a function of the Gd^{3+} concentration used.

from the transport site of the unphosphorylated Ca^{2+}-ATPase in order to examine whether Gd^{3+} physically occupied the transport site.

We followed the kinetics of dissociation of $^{45}Ca^{2+}$ bound at the transport site in the presence of either unlabeled Ca^{2+} (Figure 5A) or Gd^{3+} (Figure 5B). Figure 5A shows that isotopic exchange of the first half of the bound $^{45}Ca^{2+}$ ions was fast and its rate did not depend on the unlabeled Ca^{2+} concentration in the washing medium. On the other hand, the rate of isotopic exchange of the second half of the bound $^{45}Ca^{2+}$ ions decreased as the concentration of unlabeled Ca^{2+} ions increased. In the presence of Gd^{3+}, however, dissociation of most of the bound $^{45}Ca^{2+}$ ions from the transport site proceeded almost monophasically except for the initial, rapid phase corresponding to 1–2 nmol of $^{45}Ca^{2+}$/mg (Figure 5B). The k_{obs} value for Ca^{2+} dissociation decreased with increasing Gd^{3+} concentrations in the washing medium, 50% inhibition being obtained at a Gd^{3+} concentration less than 10 μM (Figure 5B, inset). The data clearly show that high-affinity binding of Gd^{3+} to site(s) other than the transport site inhibits dissociation of Ca^{2+} bound at the transport site.

We then examined the time courses of binding of $^{45}Ca^{2+}$ to the transport site on the unphosphorylated Ca^{2+}-ATPase preincubated with either unlabeled Ca^{2+} (Figure 6A) or Gd^{3+} (Figure 6B). When the enzyme preincubated with 30 μM unlabeled Ca^{2+} was washed with the $^{45}Ca^{2+}$-containing medium, the initial rate of $^{45}Ca^{2+}$ binding did not depend on the

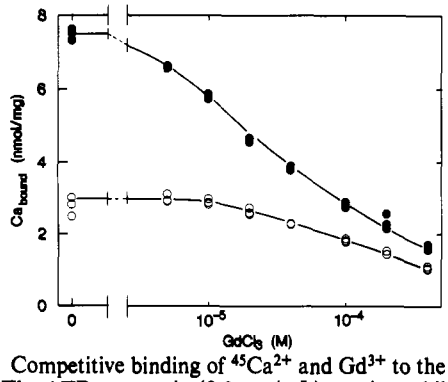


FIGURE 7: Competitive binding of $^{45}Ca^{2+}$ and Gd^{3+} to the metal-free ATPase. The ATPase protein (0.3 mg/mL) was immobilized on the filter and washed for 0.3 (○) or 2 (●) s at 6 °C with medium containing 30 mM Mops/KOH (pH 7.0), 0.3 M KCl, 2 mM $MgCl_2$, 10 mM [^{3}H]glucose, 20 μM $^{45}CaCl_2$, and 0–400 μM $GdCl_3$.

$^{45}Ca^{2+}$ concentration in the medium (Figure 6A). The total $^{45}Ca^{2+}$ binding proceeded biphasically, as expected from the time course of $^{45}Ca^{2+}$ release in the presence of unlabeled Ca^{2+} (cf. Figure 5A).

In contrast, $^{45}Ca^{2+}$ binding to the enzyme preincubated with 30 μM Gd^{3+} proceeded monophasically with k_{obs} values of 28, 35, and 49 min^{-1} in the presence of 20, 40, and 100 μM $^{45}Ca^{2+}$, respectively (Figure 6B). It is important to note that in the presence of 100 μM $^{45}Ca^{2+}$, the initial rate of $^{45}Ca^{2+}$ binding to the enzyme preincubated with 30 μM Gd^{3+} was 1.5-fold greater than that for the enzyme preincubated with the same concentration of unlabeled Ca^{2+} (compare panels A and B of Figure 6). On the other hand, $^{45}Ca^{2+}$ binding to the enzyme preincubated with neither unlabeled Ca^{2+} nor Gd^{3+} proceeded monophasically with even greater k_{obs} values of 65, 83, and 100 min^{-1} in the presence of 20, 40, and 100 μM $^{45}Ca^{2+}$, respectively (data not shown). It should be added that monophasic time courses of $^{45}Ca^{2+}$ binding were also observed when the enzyme had been preincubated with 50 or 100 μM Gd^{3+}. The inset of Figure 6B shows that the rate of $^{45}Ca^{2+}$ binding to the Gd^{3+}-treated enzyme, which was measured for 0.5 s in the presence of 20 μM $^{45}Ca^{2+}$ but in the absence of Gd^{3+}, decreased markedly as the Gd^{3+} concentration for the preincubation increased; 50% inhibition of $^{45}Ca^{2+}$ binding occurred at 20 μM Gd^{3+}.

Figure 7 shows the result of an experiment in which we measured binding of $^{45}Ca^{2+}$ to the metal-free enzyme in medium containing both $^{45}Ca^{2+}$ (20 μM) and Gd^{3+} (0–400 μM). As shown in the figure, Gd^{3+} in the $^{45}Ca^{2+}$-containing medium inhibited $^{45}Ca^{2+}$ binding. Interestingly, the Gd^{3+} concentration to give 50% inhibition was 5-fold lower when $^{45}Ca^{2+}$ binding was measured for 2 s than when it was followed for 0.3 s. This indicates that $^{45}Ca^{2+}$ bound to the enzyme at a significantly faster rate than Gd^{3+}. Another interesting point was that the $K_{1/2}$ value for the inhibitory Gd^{3+} became even lower when $^{45}Ca^{2+}$ binding was measured under the equilibrium conditions (compare Figures 4 and 7).

Discussion

Ln^{3+} Binding Sites on Ca^{2+}-ATPase. Characterization of Ln^{3+} binding sites is important because the Ln^{3+} ion has often been used as a probe to spectroscopically characterize the properties of divalent cation binding sites on the Ca^{2+}-ATPase (see the introduction). The results of this study indicate that there are several classes of binding sites for Gd^{3+} that affect the function of the enzyme.

Gd^{3+} binding to one of these sites caused inhibition of decomposition of the ADP-sensitive phosphoenzyme with an apparent dissociation constant of about 10 μM (Figure 2). This site is clearly distinct from both the transport site and the catalytic site. The Gd^{3+} ion bound at this inhibitory site could be removed by added EGTA (Figure 3B).

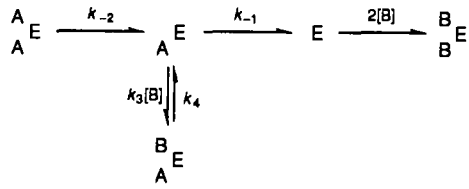
Another class of Gd^{3+} binding site is the catalytic site. We provided direct evidence that binding of GdATP at this site resulted in phosphorylation of the Ca^{2+}-ATPase when two Ca^{2+} ions were bound at the transport site (Figure 1 and Table I). We also provided evidence that Gd^{3+} ion bound at this site inhibited decomposition of the ADP-sensitive phosphoenzyme (Figure 3). These data are consistent with a conclusion by Fujimori and Jencks (1990) that LaATP phosphorylates the Ca^{2+}-ATPase to yield a phosphoenzyme that turns over slowly. It should be noted here that Gd^{3+}-dependent inhibition of $^{45}Mn^{2+}$ binding to the catalytic site appeared to occur at slightly higher concentrations of Gd^{3+} than that of P_i liberation (Figure 1). Thus, the Gd^{3+}-dependent inhibition of ATPase activity may occur mostly as a result of binding of Gd^{3+} to the site(s) other than the catalytic site.

We identified another class of binding sites for Gd^{3+} (K_d ~ 30 μM) that was involved in enhancement of NBD fluorescence in the unphosphorylated NBD-modified enzyme (Figure 4, inset). Previously we (Wakabayashi et al., 1990b) showed that Mg^{2+} increased the equilibrium level of NBD fluorescence by binding to a single site with a dissociation constant of 11 mM at pH 7.0. Analyses of Mg^{2+}-induced changes in the intrinsic tryptophan fluorescence and tryptophan to terbium fluorescence energy transfer revealed a Mg^{2+} binding site having a similar dissociation constant in the NBD-unmodified enzyme (Gullain et al., 1982; Highsmith & Head, 1983; Girardet et al., 1989). Terbium was shown to bind to the Mg^{2+} site with a dissociation constant similar to that for Gd^{3+} obtained in this study (Girardet et al., 1989). Thus, Gd^{3+} and Tb^{3+} appear to bind to the same Mg^{2+} site. This Mg^{2+} site may be highly specific to Mg^{2+}, because the Mg^{2+}-induced change in the tryptophan to terbium fluorescence energy transfer was not influenced by Ca^{2+} or ATP (Highsmith & Head, 1983; Girardet et al., 1989). The Mg^{2+} site therefore seems to be distinct from both the transport site and the catalytic site. Our previous data obtained with the NBD-labeled enzyme indicated that Mg^{2+} binding to this site stabilizes the E_1 conformation of the Ca^{2+}-free unphosphorylated enzyme (Wakabayashi et al., 1990b).

Finally, Gd^{3+} occupied binding site(s) that affected the interaction of Ca^{2+} with the transport site on the unphosphorylated Ca^{2+}-ATPase (Figures 4–7). Gd^{3+} caused parallel decreases in Ca^{2+} binding and the Ca^{2+}-dependent intrinsic fluorescence change measured at equilibrium (Figure 4). The latter effects of Gd^{3+} occurred with an apparent dissociation constant for Gd^{3+} of about 20 μM in the presence of 20 μM Ca^{2+} in the medium, suggesting that Gd^{3+} apparently competed with Ca^{2+} for the transport site with an affinity similar to that for Ca^{2+}. However, such Gd^{3+}-induced inhibitions to not necessarily mean that Gd^{3+} binds directly to the transport site. In the following paragraphs, we discuss whether Gd^{3+} occupies the transport site and whether all the Gd^{3+} binding sites affecting the interaction of Ca^{2+} with the transport site belong to a single class.

Does the Ln^{3+} Ion Bind to the Calcium Transport Site? Many investigators have already observed that Ln^{3+} ions inhibit binding of Ca^{2+} to the transport site of the unphosphorylated Ca^{2+}-ATPase (Chevallier & Butow, 1971; Meissner, 1973; Ito & Kawakita, 1984; Girardet et al., 1989; Squier et al., 1990). However, such inhibition, as noted above, does not necessarily mean that Ln^{3+} ions are able to occupy the calcium transport site with high affinity. We found (a)

Scheme I



that Gd^{3+} addition did not allow enzyme phosphorylation by GdATP to occur in the absence of added Ca^{2+} (Table I), (b) that under conditions in which we were able to detect a Ca^{2+}-induced rise of intrinsic fluorescence, Gd^{3+} did not increase the intrinsic fluorescence in the absence of added Ca^{2+} (Figure 4, inset), and (c) that Gd^{3+} added to the medium decreased the intrinsic fluorescence when the latter had already been raised by the previous Ca^{2+} addition (Figure 4). These results clearly indicate that at the transport site Gd^{3+} cannot function as an effective analogue for Ca^{2+}.

To answer a question of whether Gd^{3+} physically occupies the transport site with high affinity, we compared the effects of unlabeled Ca^{2+} and of Gd^{3+} on the kinetics of binding to and dissociation of $^{45}Ca^{2+}$ from the transport site. As shown in Figure 5A and 6A, exchange of bound $^{45}Ca^{2+}$ ions for unlabeled Ca^{2+} in the medium and vice versa exhibited biphasic time courses, with the rates of their initial phases being independent of the medium Ca^{2+} concentration and with the rates of their late phases becoming markedly slower as the medium Ca^{2+} concentration increased. These kinetics of calcium isotope exchange can be explained by the model shown in Scheme I, in which two Ca^{2+} ions react with the transport site in a sequential manner (Dupont, 1982; Petithory & Jencks, 1988). In this scheme, dissociation of bound labeled Ca^{2+} ions (A in Scheme I) in the presence of unlabeled Ca^{2+} in the medium (B in Scheme I) (cf. Figure 5A) proceeds through an intermediate ($_A$E) that partitions between rebinding of unlabeled Ca^{2+} to give ^B_AE and dissociation of the second $^{45}Ca^{2+}$ ion to give E. Alternatively, replacement of bound unlabeled Ca^{2+} ions (A in Scheme I) with $^{45}Ca^{2+}$ in the medium (B in Scheme I) (cf. Figure 6A) proceeds through intermediates $_A$E and E, with E reacting with $^{45}Ca^{2+}$ ions to form ^B_BE. The kinetic features of calcium isotope exchange described above indicate that at relatively high [Ca^{2+}] in the medium ($\gtrsim$20 μM, which were the concentrations used in this study), formation of ^B_AE from $_A$E and B was much faster than that of $_A$E from ^A_AE and that $_A$E partitioned mostly toward ^B_AE than toward E at the medium Ca^{2+} concentrations above 100 μM [cf. Petithory and Jencks (1988)].

We found that the kinetics of binding of $^{45}Ca^{2+}$ to the Gd^{3+}-treated enzyme and of dissociation of the enzyme-bound $^{45}Ca^{2+}$ in the presence of Gd^{3+} were entirely different from the kinetics of the calcium isotope exchange. (i) Binding of $^{45}Ca^{2+}$ to the enzyme preincubated with 30 μM Gd^{3+} proceeded monophasically, and its k_{obs} value increased 1.8-fold as the $^{45}Ca^{2+}$ concentration in the medium increased from 20 to 100 μM (Figure 6B). The monophasic $^{45}Ca^{2+}$ binding and the Ca^{2+}-dependent increase of its k_{obs} value were also observed when the Gd^{3+} concentration for the preincubation was increased from 30 to 100 μM, although the k_{obs} value decreased significantly (see Results and inset to Figure 6B). (ii) The initial rate of $^{45}Ca^{2+}$ binding at 100 μM $^{45}Ca^{2+}$ was 1.5-fold greater when the enzyme had been preincubated with 30 μM Gd^{3+} than when the enzyme had been preincubated with 30 μM unlabeled Ca^{2+} (compare panels A and B of Figure 6). (iii) Dissociation of most of the bound $^{45}Ca^{2+}$ ions in the presence of Gd^{3+} proceeded almost monophasically, and its k_{obs} value decreased as the Gd^{3+} concentration in the medium increased (Figure 5B). We interpreted the initial small drops in the time courses of $^{45}Ca^{2+}$ dissociation shown in Figure 5B as representing a portion of the bound $^{45}Ca^{2+}$ ions that dissociated rapidly before Gd^{3+} bound to the enzyme. This interpretation is consistent with the finding that binding of Gd^{3+} to the enzyme was relatively slow (Figure 7).

If two Gd^{3+} ions occupied the transport site with affinity similar to that for Ca^{2+}, dissociation of bound $^{45}Ca^{2+}$ ions in the presence of Gd^{3+} (Figure 5B) or binding of $^{45}Ca^{2+}$ to the enzyme preincubated with Gd^{3+} (Figure 6B) would have been described by Scheme I. Therefore, immediately after the first bound $^{45}Ca^{2+}$ ion or bound Gd^{3+} ion (A in Scheme I) left the transport site (to form $_A$E), a high concentration of Gd^{3+} or $^{45}Ca^{2+}$ in the medium (B in Scheme I) would have reacted with the empty site to form ^B_AE, thus causing a biphasic release of bound ions. The observed monophasic time courses of binding and release of $^{45}Ca^{2+}$ thus suggest that the intermediate for release of bound ions ($_A$E in Scheme I) should have partitioned entirely toward E even in the presence of 100 μM $^{45}Ca^{2+}$ (Figure 6B) or 1000 μM Gd^{3+} (Figure 5B) in the medium. Therefore, dissociation of the bound Gd^{3+} ions should have been very fast as compared to binding of $^{45}Ca^{2+}$ (Figure 6B), and binding of Gd^{3+} should have been very slow as compared to dissociation of bound $^{45}Ca^{2+}$ (Figure 5B). Our findings that the initial rate of $^{45}Ca^{2+}$ binding was greater when the enzyme had been preincubated with 30 μM Gd^{3+} than when the enzyme was preincubated with 30 μM unlabeled Ca^{2+} [see (ii) described above] and that the initial rate of $^{45}Ca^{2+}$ binding to the enzyme preincubated with Gd^{3+} increased as the medium $^{45}Ca^{2+}$ concentration increased [see (i) described above] are also consistent with the conclusion that dissociation of Gd^{3+} from the transport site is faster than binding of $^{45}Ca^{2+}$ to the transport site. In addition, the results of Figure 7 clearly show that binding of Gd^{3+} to the enzyme is slow as compared with that of Ca^{2+}. These arguments, although they are not quantitative, lead us to the conclusion that the on and off rates for Gd^{3+} are slower and faster, respectively, than the corresponding rates for Ca^{2+}. The slower on and faster off rates for Gd^{3+} do not reconcile with our assumption that the affinity of the transport site for Gd^{3+} is similar to that for Ca^{2+}.

In addition, some of the kinetic features of binding and dissociation of $^{45}Ca^{2+}$ in the presence of Gd^{3+}, namely, the monophasic time courses and the Ca^{2+} concentration dependent increase of the k_{obs} value in the case of $^{45}Ca^{2+}$ binding, are very similar to those of binding of $^{45}Ca^{2+}$ to the Ca^{2+}-free enzyme and of dissociation of the bound $^{45}Ca^{2+}$ ions into an EGTA-containing medium (see Results). These findings, together with those described above, strongly suggest that Gd^{3+} does not physically occupy the transport site with high affinity when the Ca^{2+}-ATPase is treated with Gd^{3+}.

Our view of how Gd^{3+} affects the interaction of Ca^{2+} with the transport site is visualized in Figure 8. In this figure, the calcium transport site is depicted as a binding pocket in a protein crevice in the membrane with a narrow opening toward the cytoplasm (Forbush, 1987), and Gd^{3+} ions bind to sites near the opening of the binding pocket. We speculate that binding of Gd^{3+} to these sites inhibits both the access to and release of Ca^{2+} from the binding pocket and that the extent of inhibition depends on the number of Gd^{3+} ions occupying these sites. Such inhibitions are probably due to introduction of positive charges into this region of the enzyme molecule, which would change the net surface charge. At present, however, we do not know the actual locations of these Gd^{3+} binding sites in the structure of the enzyme.

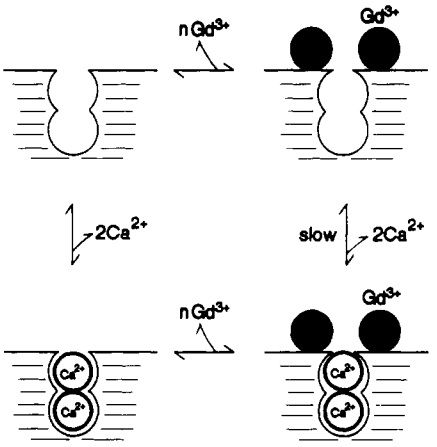


FIGURE 8: Diagram representing the interaction of Ca^{2+} and Gd^{3+} with binding sites on the Ca^{2+}-ATPase.

Our conclusion that Gd^{3+} does not bind to the calcium transport site of the Ca^{2+}-ATPase with high affinity does not fit with the widely held view that the Ln^{3+} ion binds to the Ca^{2+} binding site and acts as a very effective Ca^{2+} analogue. However, an example of the inability of Ln^{3+} ions to replace Ca^{2+} has been reported previously. Chantler (1983) presented evidence that Ln^{3+} cannot compete with Ca^{2+} for the Ca^{2+}-specific site of scallop myosin.

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