Ca\(^{2+}\) Binding to Sarcoplasmic Reticulum ATPase Revisited

II. EQUILIBRIUM AND KINETIC EVIDENCE FOR A TWO-ROUTE MECHANISM*

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The experiments reported in the present paper were designed to check the model proposed for Ca\(^{2+}\) binding in the preceding paper (Forge, V., Mintz, E., and Guillain, F. (1993) J. Biol. Chem. 268, 10953–10960). The pH dependence of the Mg\(^{2+}\)-induced variation of the intrinsic fluorescence, as well as that of the phosphorylation by P, confirmed that there are several species of Ca\(^{2+}\)-deprived ATPase.

Kinetics of Ca\(^{2+}\) binding as a function of pH suggested that the deprotonated form of the ATPase binds Ca\(^{2+}\) rapidly (k > 50 s\(^{-1}\)), whereas the protonated forms bind Ca\(^{2+}\) slowly (1.3–2.7 s\(^{-1}\)). At variance with other models which are linear, slow and rapid Ca\(^{2+}\) binding take two different routes, and intermediate pH values and Mg\(^{2+}\), which favors the deprotonated forms, result in biphasic kinetics.

Mg\(^{2+}\) binds to all Ca\(^{2+}\)-deprived species and to species having one bound Ca\(^{2+}\) but does not bind to ECa\(_0\). This is the reason why Mg\(^{2+}\) inhibits Ca\(^{2+}\) binding, and this inhibition is removed in the presence of adenosine-5’-O-(3-thiotriphosphate) which drives Mg\(^{2+}\) into the catalytic site.

Ca\(^{2+}\)-ATPase is a membranous enzyme which pumps Ca\(^{2+}\) from the cytoplasm into the sarcoplasmic reticulum lumen, at the expense of ATP hydrolysis. Cytoplasmic Ca\(^{2+}\) binding at the transport sites is a crucial step in the ATPase cycle, as it induces a change in the chemical reactivity of the ATPase catalytic site. In the absence of Ca\(^{2+}\), ATPase is phosphorylatable by P, whereas in the presence of Ca\(^{2+}\), it is phosphorylatable by ATP. Once the phosphoenzyme has been formed from ATP, the Ca\(^{2+}\)-bound at the transport sites can be released into the lumen. In both phosphorylation reactions, Mg\(^{2+}\) is necessary for the covalent phosphoenzyme formation, as the substrate for phosphorylation by ATP is Mg-ATP (Vianna, 1975), and the phosphorylation by P, starts from the ternary complex ATPase-Mg-Pi (Punzengruber et al., 1978).

As Ca\(^{2+}\) binding at the transport sites induces a change in the chemical reactivity of the catalytic site, and as the orientation of the transport sites is changed upon phosphorylation, Ca\(^{2+}\) binding is thought to induce at least one conformational change. Whether this conformational change occurs prior to, or during, the Ca\(^{2+}\) binding process is still being discussed. According to de Meis and Vianna (1979), there are two main conformations. One conformation, E\(_2\) or E*, displays inward-oriented low affinity binding sites, and the other one, E\(_1\) or E, displays outward-oriented high affinity binding sites. A pH-driven E\(_1\)-E\(_2\) equilibrium involving one proton and preceding Ca\(^{2+}\) binding has been described by Pick and Karlis (1982) and more recently by Wakabayashi et al. (1990), who suggested that two protons were involved. Kinetic studies from different laboratories have led to a description of Ca\(^{2+}\) binding as a biphasic process, comprising a fast binding of a first Ca\(^{2+}\) ion followed by a slow conformational change which allows the binding of a second Ca\(^{2+}\) (Inesi et al., 1986; Guillain et al., 1981; Dupont, 1982; Champeil et al., 1983). The existence of a fast phase depends on the conditions: it is favored by the presence of Mg\(^{2+}\), by neutral or alkaline pH, and by low temperatures (Guillain et al., 1981; Dupont, 1982; Champeil et al., 1983; Dupont, 1984; Nakamura, 1989; Moutin and Dupont, 1991), and there is still no clear explanation of how Mg\(^{2+}\), H\(^{+}\), or temperature make it possible to separate the two Ca\(^{2+}\) pools. In addition, isotopic exchange of bound Ca\(^{2+}\) clearly shows the existence of two Ca\(^{2+}\) pools (Dupont, 1982).

A few studies have suggested that H\(^{+}\) and Ca\(^{2+}\) were countertransported by ATPase (Madeira, 1980; Chiesi and Inesi, 1980; Meissner, 1981). More recently, Lévy et al. (1990) showed such a countertransport with a 3H\(^{+}\)/2Ca\(^{2+}\) stoichiometry, which confirms the observation by Yamagushi and Kanazawa (1984, 1985) that three H\(^{+}\) are exchanged for two Ca\(^{2+}\) at each step of Ca\(^{2+}\) binding or dissociation during the transport cycle. It is thus likely that protons are involved in the Ca\(^{2+}\) binding or dissociation process and consequently modify the kinetics of Ca\(^{2+}\) binding or dissociation.

Equilibrium measurements of Ca\(^{2+}\) binding at various pH values and Mg\(^{2+}\) concentrations have led us to describe Ca\(^{2+}\) binding as shown in Scheme 1. According to this model, there

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![Scheme 1](image-url)

**Scheme 1**. Ca\(^{2+}\) binding at equilibrium and its modulation by H\(^{+}\) and Mg\(^{2+}\) as described in the previous paper (Forge et al., 1993). The simulations reported here were done using the following dissociation constants: K\(_1\) = 0.1 μM, K\(_2\) = 0.1 μM for Ca\(^{2+}\), L\(_0\) = 0.15 μM, L\(_1\) = 0.012 μM, L\(_2\) = 0.012 μM for H\(^{+}\), and M\(_0\) = 4 mM, M\(_1\) = 250 mM, M\(_2\) = 20 mM, M\(_3\) = 0.4 mM, M\(_4\) = 1.2 mM, M\(_5\) = 0.8 mM, M\(_6\) = 10 mM, M\(_7\) = 0.8 mM for Mg\(^{2+}\).
are three crucial H⁺ in the Ca²⁺ binding process. The process starts with the dissociation of two H⁺. Then, there are two alternatives; one route requires the dissociation of the third H⁺ prior to the binding of the first Ca²⁺, whereas the other route allows Ca²⁺ binding to protonated ATPase. Whatever the route taken, the binding of the second Ca²⁺ requires the dissociation of the third H⁺ ion. Mg²⁺ binds to all species, except to EC₃, which has saturated transport sites. Binding of the second Ca²⁺ requires the dissociation of both H⁺ and Mg²⁺. The overall effect of Mg²⁺ is to favor the deprotonation of ATPase.

This model was built to be the simplest model allowing us to interpret the effects of H⁺ and Mg²⁺ on equilibrium Ca²⁺ binding. It describes the minimum number of H⁺ and Mg²⁺ interfering with Ca²⁺ binding, so that binding sites which would not affect Ca²⁺ binding are not described.

Experiments reported here were designed to check the model and analyze the different species described in the model in terms of their existence and role during the ATPase cycle. Particular attention was paid to the following points: (i) the consequences of the proton-driven equilibria on phosphorylation by P₁ and on Mg²⁺ binding in the absence of Ca²⁺; (ii) the existence of the EHCa species; (iii) the consequences of the existence of two routes on the kinetics of Ca²⁺ binding; (iv) the apparent contradiction between the exclusion of Me²⁺ interfering with Ca²⁺ binding are not described.

RESULTS

Existence of a Route Involving One H⁺ between the First and the Second Ca²⁺ Dissociation—A decisive addition was made to our knowledge of the Ca²⁺ binding mechanism when Dupont (1982) showed that once bound, the two Ca²⁺ are not exchangeable in an equivalent way. Fig. 1 shows Ca²⁺ dissociation in the presence of an excess of EGTA and isotopic exchange at various concentrations of [⁴Ca]Ca²⁺. These experiments were done in the absence of Mg²⁺ and at different pH values. ATPase was incubated with just enough [⁴Ca]Ca²⁺ to saturate the transport sites and to avoid non-specific Ca²⁺ binding (see "Materials and Methods"). Under all conditions studied here, starting from EC₃, both Ca²⁺ ions dissociated rapidly in the presence of an excess of EGTA. When dissociation was achieved in the presence of [⁴Ca]Ca²⁺, half of the bound

1 The abbreviations used are: SR, sarcoplasmic reticulum; Mops, 4-morpholino-ethanesulfonic acid; Mes, 2-(N-morpholino)ethanesulfonic acid; Tes, N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; AMPPCP, adenylyl-(5'-methylene)imidophosphonate; ATP-γ-S, adenosine-5'-O(3-thiotriphosphate).
$[^{45}\text{Ca}]\text{Ca}^{2+}$ was rapidly replaced by $[^{40}\text{Ca}]\text{Ca}^{2+}$ from the medium with a rate constant of $5 \text{ s}^{-1}$. This rapidly exchangeable $\text{Ca}^{2+}$ has been identified as the last $\text{Ca}^{2+}$ bound to ATPase (Inesi, 1987; Petithory and Jencks, 1988a) and therefore to the second $\text{Ca}^{2+}$ in Scheme 1. The other half of the bound $[^{40}\text{Ca}]\text{Ca}^{2+}$ displayed a dissociation rate constant which diminished when the free $[^{40}\text{Ca}]\text{Ca}^{2+}$ concentration in the medium was increased. This effect of $[^{40}\text{Ca}]\text{Ca}^{2+}$ was explained by the binding of $[^{40}\text{Ca}]\text{Ca}^{2+}$ at the second site, impairing dissociation of half of the bound $[^{40}\text{Ca}][\text{Ca}^{2+}]$. As the dissociation of this slowly exchangeable $\text{Ca}^{2+}$ is impaired by the binding of $\text{Ca}^{2+}$ at the second site, it has been identified as the first $\text{Ca}^{2+}$ bound to ATPase (Petithory and Jencks, 1988a).

The dependence of the observed rate constant of the slow phase on the free $[^{40}\text{Ca}]\text{Ca}^{2+}$ concentration was plotted at different pH (Fig. 2). The curves were noncooperative ($n_H = 1$) and allowed evaluation of the apparent affinity of the rapidly exchangeable $\text{Ca}^{2+}$ from $\text{Ca}_{12}$, the $\text{Ca}^{2+}$ concentration corresponding to the half-maximum rate constant. This affinity was found higher than the overall affinity for $\text{Ca}^{2+}$ deduced from binding at equilibrium under all conditions explored here, namely in the absence of Mg$^{2+}$ and at pH 6, 6.3, and 7, confirming the results of Orlowski and Champel (1991) at pH 6 and in the presence of 20 mM Mg$^{2+}$. The apparent affinity of the rapidly exchangeable $\text{Ca}^{2+}$ varied with pH and its dependence on the H$^+$ concentration was linear (inset in Fig. 2), confirming that the binding of the second $\text{Ca}^{2+}$ is inhibited by one H$^+$. In addition, the fact that the first $\text{Ca}^{2+}$ bound, i.e. the slowly exchangeable $\text{Ca}^{2+}$, has a higher dissociation rate constant at acidic pH than at alkaline pH means that it dissociates from the EHCa species. If this were not the case, EHCa would be a dead end complex and accumulate at acidic pH, resulting in a decrease in the observed dissociation rate constant of the slowly exchangeable $\text{Ca}^{2+}$. As our measurements show an increase in this rate constant, they confirm the pattern with two possible routes depending on the pH as proposed in the preceding paper (Forge et al., 1993).

Mg$^{2+}$ Binding as a Function of pH—The left part of Scheme 1 describes H$^+$ and Mg$^{2+}$-dependent equilibria in the absence of $\text{Ca}^{2+}$. This part of Scheme 1 may be well illustrated by the variations of the intrinsic fluorescence of ATPase ($AF/F$) induced by Mg$^{2+}$ at various pH. The changes in the intrinsic fluorescence of ATPase upon $\text{Ca}^{2+}$ and Mg$^{2+}$ additions have already been used to study the inhibition of $\text{Ca}^{2+}$ binding by Mg$^{2+}$ (Guillain et al., 1982). Mg$^{2+}$ has been found to induce a blue shift in the fluorescence emission spectrum, and 315 nm was determined to be a suitable emission wavelength to study Mg$^{2+}$ binding.

Fig. 3 presents the fluorescence increase due to the presence of 30 mM MgCl$_2$ at various pH. At each pH, ATPase was incubated in 2 mM EGTA, and the intrinsic fluorescence increase upon addition of 3 mM Ca$^{2+}$ was measured in the absence and in the presence of 30 mM Mg$^{2+}$. As the Ca$^{2+}$-induced fluorescence change is insensitive to pH (Guillain et al., 1982), the fluorescence increase due to Mg$^{2+}$ was evaluated by difference. At pH 6, ATPase fluorescence was not sensitive to Mg$^{2+}$, and maximum sensitivity was reached at pH 7.5. The steep slope of the curve as regards the H$^+$ concentration confirmed that several H$^+$ are involved in the Mg$^{2+}$-binding process (Fig. 3). The dashed line in Fig. 3 is the best fit to the fluorescence variations with H$^+$ concentration using the Hill equation. An estimated cooperativity of 2 confirms that at least two H$^+$ are involved.

Phosphorylation by P$_i$ as a Function of pH—A second illustration of Scheme 1 in the absence of $\text{Ca}^{2+}$ is given by phosphorylation of ATPase by P$_i$. Phosphorylation by P$_i$ is known to require millimolar concentrations of P$_i$ and Mg$^{2+}$ and to be favored by acidic pH (Kanazawa and Boyer, 1973; Masuda and de Meis, 1973). ATPase phosphorylation by P$_i$ was measured by $[^{25}\text{P}]$P$_i$ incorporation in the presence of 5 mM $[^{32}\text{P}]$P$_i$ and 30 mM MgCl$_2$ (Fig. 4). The phosphoenzyme level increased from 0.3 nmol/mg at pH 8 to 3.25 nmol/mg at pH 6, which was the highest level of phosphoenzyme obtained under these conditions. Here again, the steepness of the H$^+$ dependency of the observed rate constant of the slow phase on the free $[^{40}\text{Ca}]\text{Ca}^{2+}$ concentration was plotted at different pH (Fig. 2). The curves were noncooperative ($n_H = 1$) and allowed evaluation of the apparent affinity of the rapidly exchangeable $\text{Ca}^{2+}$ from $\text{Ca}_{12}$, the $\text{Ca}^{2+}$ concentration corresponding to the half-maximum rate constant. This affinity was found higher than the overall affinity for $\text{Ca}^{2+}$ deduced from binding at equilibrium under all conditions explored here, namely in the absence of Mg$^{2+}$ and at pH 6, 6.3, and 7, confirming the results of Orlowski and Champel (1991) at pH 6 and in the presence of 20 mM Mg$^{2+}$. The apparent affinity of the rapidly exchangeable $\text{Ca}^{2+}$ varied with pH and its dependence on the H$^+$ concentration was linear (inset in Fig. 2), confirming that the binding of the second $\text{Ca}^{2+}$ is inhibited by one H$^+$. In addition, the fact that the first $\text{Ca}^{2+}$ bound, i.e. the slowly exchangeable $\text{Ca}^{2+}$, has a higher dissociation rate constant at acidic pH than at alkaline pH means that it dissociates from the EHCa species. If this were not the case, EHCa would be a dead end complex and accumulate at acidic pH, resulting in a decrease in the observed dissociation rate constant of the slowly exchangeable $\text{Ca}^{2+}$. As our measurements show an increase in this rate constant, they confirm the pattern with two possible routes depending on the pH as proposed in the preceding paper (Forge et al., 1993).

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dependence of the phosphoenzyme level indicated that several H+ were involved in the phosphorylation by Pi. This pH dependence, together with the fact that millimolar Mg2+ is required in order to phosphorylate, suggested E3H as a candidate for the species to be phosphorylated by Pi.

Ca2+ Binding Kinetics at 20 °C—Starting from data gathered at equilibrium in the previous paper (Forge et al., 1993), we have performed a systematic analysis of the kinetics of Ca2+ binding at the transport sites by measurements of both (ΔF/F), the Ca2+-induced intrinsic fluorescence change, and [45Ca]Ca2+ binding. For simplicity and to emphasize H+ and Mg2+ effects, we present here Ca2+ binding kinetics studied at the saturating Ca2+ concentration of 100 μM, at various pH and in the absence or presence of 30 mM Mg2+. Fig. 5 shows the Ca2+ binding kinetics obtained at different pH in the absence of Mg2+ (Fig. 5A) and in the presence of 30 mM Mg2+ (Fig. 5B). In the absence of Mg2+, nonspecific Ca2+ binding impaired radioactive measurements (see preceding paper (Forge et al., 1993)). Thus, under these conditions with native vesicles, fluorescence was the preferred method. Nevertheless, in the presence of Mg2+, both fluorescence and radioactive methods gave similar results, allowing direct interpretation of the kinetics obtained by fluorescence. Fig. 5 shows that depending on the pH and on the presence of Mg2+, the kinetics of Ca2+ binding were mono- or biphasic.

The effect of pH on the kinetics of Ca2+ binding in the absence of Mg2+ can be summarized as follows. (i) At pH 6, the Ca2+ binding kinetics were monophasic with an observed rate constant of 1.3 s⁻¹. (ii) At pH 7, the kinetics were still monophasic with an observed rate constant of 2.7 s⁻¹. (iii) At pH 8, the kinetics were biphasic; a slow phase with an observed rate constant of 2.7 s⁻¹ accounted for 60% of the total amplitude, and a fast phase with an observed rate constant higher than 50 s⁻¹ accounted for the remaining 40%. (iv) At pH 9, the kinetics were fast and seemed monophasic with an observed rate constant higher than 50 s⁻¹. We could not measure any rate constants due to the time response of our stopped-flow apparatus.

The rapid phase appeared at alkaline pH and thus seemed to be correlated with enzyme deprotonation. According to Scheme 1, the Ca2+-deprived ATPase resides in three species, E3H, EH, and E, the distribution of which depends on pH; at pH 6, all ATPase is in the E3H form, whereas at pH 9, all ATPase is in the E form (see previous paper (Forge et al., 1993)). When compared with the predictions of Scheme 1, the proportion of the signal corresponding to the rapid phase of Ca2+ binding was found to parallel the proportion of E (Fig. 6). This suggested that Ca2+ binding was slow when the protonated species E3H or EH were predominant before Ca2+ addition and that it was fast when the deprotonated species E was predominant. According to Scheme 1, there is no E3H3Ca species, and thus we had to assume slow dissociation for protons. This assumption was verified by measurements of Ca2+ binding kinetics together with a pH jump from pH 6 or 7 to 9 (Fig. 7). The objective for carrying out such pH jumps was to compare the rates of proton dissociation and Ca2+ binding. The kinetics observed were biphasic and similar, whether the jump was from pH 6 to 9 or from pH 7 to 9. Note that the amplitude of the slow phase (dashed line in Fig. 7) was equal to the total amplitude of all the Ca2+ binding signals reported here in the absence of a pH jump. We therefore attributed the small rapid phase to pH equilibration following the mixing. The slow phase (10 s⁻¹) was slower than the kinetics measured at pH 9 without a pH jump (Fig. 5A), as expected from the assumption that H+ dissociation was a rate-limiting step.

When associated with pH jumps, the kinetics were faster than the slow phases observed at pH 6 (1.3 s⁻¹) and pH 7 (2.7 s⁻¹), indicating that H+ association could also modify Ca2+ binding kinetics. For example, according to Scheme 1, both H+ and Ca2+ can bind to EH, so that H+ binding could slow down Ca2+ binding by competition. This could also be the reason why the kinetics were slower at pH 6 than at pH 7.

Fig. 5B shows kinetics of Ca2+ binding in the presence of 30 mM Mg2+ measured either by intrinsic fluorescence or by [45Ca]Ca2+ incorporation. At pH 6, the kinetics were monophasic and slow (1.3 s⁻¹). At pH 7, the kinetics were biphasic with a slow rate constant of 4 s⁻¹, and at pH 8, the kinetics...
that ATPase has to lose the bound Me prior to binding the rate constant of Guillain for the slow phase observed at pH 8. Note that the observed second Ca\(^{2+}\), and this Me dissociation could be responsible for the rapid phase. The relatively slow phase appearing at pH 8 in the absence of Ca\(^{2+}\) binding in the absence of Me could be due to the Me bound to the rapid phase. The reason for the inhibition of Ca\(^{2+}\) binding in the presence of Mg\(^{2+}\) must be looked for at other sites. Because Mg\(^{2+}\) is necessary for both phosphorylations by ATP and P\(_i\), it is assumed to bind to the catalytic site. Nevertheless, it should not bind uniformly to all species appearing in the Ca\(^{2+}\) binding process, otherwise no inhibition of Ca\(^{2+}\) binding would be evidenced. The main reason for the observed inhibition of Ca\(^{2+}\) binding in the presence of Mg\(^{2+}\) would be the fact that ECa\(_2\) does not bind Mg\(^{2+}\), as evidenced at equilibrium in the preceding paper (Forge et al., 1993).

### FIG. 6. pH dependence of the amplitude of the fast phase of Ca\(^{2+}\) binding in the absence (○) and in the presence (△) of 30 mM Mg\(^{2+}\). The dashed lines show the population of deprotonated ATPase as predicted by Scheme 1.

![Deprotonated ATPase](image)

were also biphasic, and the slow rate constant was 4 s\(^{-1}\). At both pH 7 and 8, the fast component was too fast to allow measurement of its rate constant. According to Scheme 1 and its predictions at equilibrium (see preceding paper (Forge et al., 1993), the main effect of Mg\(^{2+}\), in the absence of Ca\(^{2+}\), is to favor the pool of deprotonated species E + EMg + EMg\(_2\), leading to 50% of ATPase in a deprotonated form at pH 7 and 100% at pH 8 (Fig. 6). At variance with measurements in the absence of Mg\(^{2+}\), the proportion of ATPase in a deprotonated form could not be correlated with the amplitude of the rapid phase. The relatively slow phase appearing at pH 8 in the presence of Mg\(^{2+}\) could be due to the Mg\(^{2+}\) bound to the deprotonated enzyme. According to measurements at equilibrium, there is no species having bound Mg\(^{2+}\) plus two Ca\(^{2+}\), i.e. the ECa\(_2\) species does not bind Mg\(^{2+}\), although there are species having bound Mg\(^{2+}\) plus one Ca\(^{2+}\). Thus, it appears that ATPase has to lose the bound Mg\(^{2+}\) prior to binding the second Ca\(^{2+}\), and this Mg\(^{2+}\) dissociation could be responsible for the slow phase observed at pH 8. Note that the observed rate constant of 4 s\(^{-1}\) could represent the Mg\(^{2+}\) dissociation rate constant of 20 s\(^{-1}\) measured in the absence of Ca\(^{2+}\) by Guillain et al. (1982), modulated by the presence of 30 mM Mg\(^{2+}\) in the medium.

### FIG. 7. Kinetics of Ca\(^{2+}\) binding associated with a pH jump. SR vesicles were equilibrated with EGTA in a 10 mM Mes-Tris buffer at pH 6 and mixed with Ca\(^{2+}\) in a 200 mM Tris-HCl buffer at pH 9, to yield a final concentration of 100 μM Ca\(^{2+}\) at pH 9.

![Kinetics of Ca\(^{2+}\) binding](image)

Presence of Ca\(^{2+}\)—To verify whether or not Mg\(^{2+}\) competes for Ca\(^{2+}\) on the ECa species by binding to the second Ca\(^{2+}\) site, we compared Ca\(^{2+}\) dissociation induced by an excess of EGTA in the absence of Mg\(^{2+}\) and in the presence of 50 mM Mg\(^{2+}\). Indeed, if Mg\(^{2+}\) was to bind to the second Ca\(^{2+}\) site, this would impair the dissociation of the first Ca\(^{2+}\), as a high concentration of 40Ca\(^{2+}\) does (see Fig. 1). Neither at pH 6, nor at pH 7 (Fig. 8) or 8, did the presence of 50 mM MgCl\(_2\) during Ca\(^{2+}\) dissociation cause a difference when compared with the control experiment, except for a slight acceleration at pH 6. Note that the presence of 50 mM Mg\(^{2+}\) in the incubation medium did not change the kinetics. Thus, Mg\(^{2+}\) did not bind to the second Ca\(^{2+}\) site. Isotopic exchange experiments with 1 mM 40Ca\(^{2+}\) were performed during the same set of experiments. They are reported here for comparison, and show the unambiguous difference between an exchange on the rapidly exchangeable Ca\(^{2+}\) site and an EGTA-induced Ca\(^{2+}\) dissociation.

The experiments reported in Fig. 8 show that Mg\(^{2+}\) has no effect on the dissociation of the first Ca\(^{2+}\) ion and consequently that it does not bind to the second transport site. Thus, the reason for the inhibition of Ca\(^{2+}\) binding in the presence of Mg\(^{2+}\) must be looked for at other sites. Because Mg\(^{2+}\) is necessary for both phosphorylations by ATP and P\(_i\), it is assumed to bind to the catalytic site. Nevertheless, it should not bind uniformly to all species appearing in the Ca\(^{2+}\) binding process, otherwise no inhibition of Ca\(^{2+}\) binding would be evidenced. The main reason for the observed inhibition of Ca\(^{2+}\) binding in the presence of Mg\(^{2+}\) would be the fact that ECa\(_2\) does not bind Mg\(^{2+}\), as evidenced at equilibrium in the preceding paper (Forge et al., 1993).

To confirm this, we looked for conditions liable to cause Mg\(^{2+}\) binding to all species, including ECa\(_2\), which would, as a consequence, reduce or even cancel out the inhibition of Ca\(^{2+}\) binding. Bearing in mind that the substrate for phosphorylation of ATPase in the presence of Ca\(^{2+}\) is the Mg-ATP complex (Vianna, 1975), we looked for an ATP analog which induces Mg\(^{2+}\) binding to ECa\(_2\), without impairing binding at equilibrium, namely, without inducing ATPase phosphorylation.

In the absence of Ca\(^{2+}\), Mg\(^{2+}\) is known to bind to ATPase with an apparent dissociation constant of 5 mM at pH 7.2, which is shifted to 30 μM in the presence of 100 μM ATP (Lacapere et al., 1990). The property of ATP to increase the affinity for Mg\(^{2+}\) was taken as a criterion to select an ATP

### FIG. 8. Effect of high Mg\(^{2+}\) concentration on the kinetics of Ca\(^{2+}\) dissociation at pH 7. SR vesicles were equilibrated with 10 μM free 14Ca\(^{2+}\). The perfusate contained 0.8 mM EGTA (○), 2.3 mM EGTA plus 50 mM Mg\(^{2+}\) (△), and 1 mM 40Ca\(^{2+}\) (○).

![Effect of high Mg\(^{2+}\) concentration](image)
analog which displays the same property in the presence of Ca$^+$ and thus drives Mg$^+$ ions into the catalytic site.

The apparent dissociation constant of Mg$^{2+}$ was measured by the variation of ATPase intrinsic fluorescence upon Mg$^{2+}$ addition, in the absence and in the presence of nucleotides, as in Lacapère et al. (1990). As expected, the dissociation constant of Mg$^{2+}$ was shifted from 5 mM to 100 $\mu$M in the presence of 100 $\mu$M ATP (Fig. 9). This was repeated in the presence of AMPPCP and ATP$\gamma$S. AMPPCP did not affect the dissociation constant for Mg$^{2+}$, whereas 100 $\mu$M ATP$\gamma$S shifted the apparent dissociation constant for Mg$^{2+}$ to 300 $\mu$M. Therefore, ATP$\gamma$S was a good candidate to modify the inhibition of Ca$^{2+}$ binding in the presence of Mg$^{2+}$, whereas AMPPCP was expected not to affect Ca$^{2+}$ binding.

Ca$^{2+}$ binding was measured at equilibrium, as described in the preceding paper (Forge et al., 1993), in the presence of 3 mM Mg$^{2+}$, 300 $\mu$M AMPPCP or ATP$\gamma$S, or Mg$^{2+}$ plus nucleotide (Fig. 10). Compared with the control conditions, the presence of 3 mM Mg$^{2+}$ induced an inhibition of Ca$^{2+}$ binding revealed by the shift of the binding curve toward the higher free Ca$^{2+}$ concentrations. In the absence of Mg$^{2+}$, both nucleotides induced a small enhancement in the apparent affinity for Ca$^{2+}$. In the presence of Mg$^{2+}$, addition of AMPPCP had no effect on Ca$^{2+}$ binding, as shown by the unchanged Ca$^{2+}$ binding curve (Fig. 10A). Conversely to AMPPCP, ATP$\gamma$S cancelled out the inhibition due to the presence of 3 mM Mg$^{2+}$ (Fig. 10B).

ATP$\gamma$S is known to be a poor substrate for ATPase activity. There is a residual activity which accounts for 0.5% of the activity measured with ATP as substrate (Yasuoka et al., 1982). Under the conditions used in the experiments of Fig. 10, ATPase phosphorylation by [35S]ATP$\gamma$S was slow (half-life, 1–3 min; data not shown) and, therefore, did not interfere here with Ca$^{2+}$ binding measurements.

Therefore, Fig. 10 shows that in the presence of ATP$\gamma$S, Mg$^{2+}$ binds uniformly to all species appearing in the Ca$^{2+}$ binding process and thus cancels out the inhibition due to the presence of Mg$^{2+}$ alone. This confirms that Mg$^{2+}$ alone does not bind to the ECa$^2_0$ species.

**DISCUSSION**

*Effect of H$^+$ and Mg$^{2+}$ on the Kinetics of Ca$^{2+}$ Binding and Dissociation*—A pH-driven E$_2$E$_1$ equilibrium preceding Ca$^{2+}$ binding has been described by Pick and Karlish (1982), Froud and Lee (1986), and Wakabayashi et al. (1990). In these reports, acidic pH was shown to favor the E$_2$ conformation of ATPase. Ca$^{2+}$ binding measurements at equilibrium have led us to assume that three H$^+$ were involved in the pH-dependent equilibrium in the absence of Ca$^{2+}$. This was confirmed here via the pH dependence of the intrinsic fluorescence sensitivity to Mg$^{2+}$ (Fig. 3) and by the pH dependence of the phosphorylation by ATP$\gamma$S (Fig. 4). Wakabayashi et al. (1990) also suggested that in the absence of Ca$^{2+}$, Mg$^{2+}$ could drive the enzyme in a deprotonated conformation. This effect of Mg$^{2+}$ is implicitly contained in Scheme 1, as the affinity of the deprotonated species for Mg$^{2+}$ is higher than that of the protonated species.

The kinetics of Ca$^{2+}$ binding have been described by different schemes, depending on the step where a conformational change occurs. According to Wakabayashi et al. (1990), Ca$^{2+}$ binding is monophasic and limited by a pH-driven conformational change occurring prior to Ca$^{2+}$ binding, as initially proposed by Pick and Karlish (1982). Other laboratories have described Ca$^{2+}$ binding as a biphasic process comprising a fast binding of a first Ca$^{2+}$ ion followed by a slow conformational change which allows binding of a second Ca$^{2+}$ (Inesi et al., 1980; Guillain et al., 1981; Dupont, 1982; Champel et al., 1983). A possible explanation for the monophasic kinetics reported by Wakabayashi et al. (1990) is that these authors used an enzyme labeled with 4-nitrobenzo-2-oxa-1,3-diazole, a pH-sensitive fluorescence probe, which would, according to our model, respond to the H$^+$ dissociation steps preceding Ca$^{2+}$ binding. The same explanation holds for Pick and Karlish (1982), as fluorescein 5'-isothiocyanate is also a pH-sensitive fluorescence probe, which would respond to the pH-dependent steps upon Ca$^{2+}$ addition (see their Fig. 4).

At variance with the results cited above, and because we had constructed a scheme from equilibrium studies, we were able to predict the initial state of ATPase at each pH. The
knowledge of the initial state allowed us to interpret differently the kinetics of Ca\textsuperscript{2+} binding. The main feature in our interpretation is the existence of two routes for Ca\textsuperscript{2+} binding. One route starts with the protonated species and is slow, because it involves one or two H\textsuperscript{+} dissociation steps, which were shown by pH jump experiments to be rate-limiting steps. The other route starts with the deprotonated species and is fast. According to this scheme, at acidic pH the kinetics of Ca\textsuperscript{2+} binding is monophasic and slow whereas, at alkaline pH, it is monophasic and fast. For intermediate pH values, for example at pH 8, and in the absence of Mg\textsuperscript{2+}, the biphasic behavior does not arise from a sequential binding of two Ca\textsuperscript{2+} ions but rather from the initial distribution of ATPase which is half in EH and half in E and thus takes both routes simultaneously.

Mg\textsuperscript{2+} was found to modify the Ca\textsuperscript{2+} binding kinetics (Fig. 5). Champeil et al. (1983) and Moutin and Dupont (1991) reported that at pH 7 the kinetics of the intrinsic fluorescence increase upon Ca\textsuperscript{2+} binding were biphasic in the presence of Mg\textsuperscript{2+}. This effect of Mg\textsuperscript{2+} was confirmed here by [\textsuperscript{45}Ca]Ca\textsuperscript{2+} measurements and at various pH values (Fig. 5) and could be predicted from Scheme 1, as Mg\textsuperscript{2+} shifts the distribution of ATPase toward deprotonated species (see Fig. 6 in the previous paper (Forge et al., 1993). At pH 7 and in the absence of Mg\textsuperscript{2+}, 100% of ATPase is protonated, i.e. in EH and EH, and thus Ca\textsuperscript{2+} binding takes the slow route, whereas in the presence of 30 mM Mg\textsuperscript{2+} 50% of ATPase is deprotonated, i.e. in E, EMg, and EMg\textsubscript{2}. In this case, 50% of ATPase takes the fast route, inducing a biphasic kinetics (Fig. 6).

Following this reasoning, the kinetics of Ca\textsuperscript{2+} binding in the presence of Mg\textsuperscript{2+} should be monophasic and fast at pH 8, because all ATPase is deprotonated under these conditions (Fig. 6). Nevertheless, because there is no species having bound two Ca\textsuperscript{2+} ions plus Mg\textsuperscript{2+}, as demonstrated in the preceding paper (Forge et al., 1993), Mg\textsuperscript{2+} should dissociate to make it possible for the second Ca\textsuperscript{2+} to bind. Thus, the kinetics were not monophasic and a slow phase appeared, which has been attributed to the dissociation of Mg\textsuperscript{2+}.

At variance with the other schemes derived from previous studies of Ca\textsuperscript{2+} binding kinetics, Scheme 1 is not linear. The two routes described here point out the fact that dissociation of the three protons involved in the Ca\textsuperscript{2+} binding process does not necessarily occur prior to Ca\textsuperscript{2+} binding. On the contrary, one route makes it possible for ATPase to bind the first Ca\textsuperscript{2+} on a protonated species and the existence of a "mixed" species EHCa has been demonstrated by the pH dependence of the isotopic exchange (Figs. 1 and 2). Finally, the main difference with other interpretations resides in the fact that the limiting steps in the Ca\textsuperscript{2+} binding kinetics at 100 \mu M Ca\textsuperscript{2+}, a saturating concentration, are the H\textsuperscript{+} dissociation steps and/or the Mg\textsuperscript{2+} dissociation steps. Note also that "the burst of Ca\textsuperscript{2+} binding in presence of high [Ca\textsuperscript{2+}]" observed by Petithory and Jencks (1988b) at pH 7 and in the presence of 5 mM Mg\textsuperscript{2+} could be due to the nonnegligible fraction of deprotonated ATPase predicted from Scheme 1.

Interaction between Catalytic Site and Transport Sites: Role of Mg\textsuperscript{2+}—During the ATPase cycle, the Ca\textsuperscript{2+} binding step as a whole drives ATPase from a species which can be phosphorylated by P, to a species which can be phosphorylated by ATP. Thus, going from EH3 to ECa2, ATPase undergoes changes in its chemical specificity, depending on the presence of Ca\textsuperscript{2+} at the transport sites. In this regard, among the numerous H\textsuperscript{+} of the 1001 amino acids of ATPase, the three protons involved in the Ca\textsuperscript{2+} binding process probably also participate in the chemical specificity of ATPase. In line with this is the fact that phosphorylation by P\textsubscript{i} is favored by acidic pH. EH\textsubscript{3} is thus the best candidate for the species which is phosphorylatable by P\textsubscript{i}, and at the other end, ECa\textsubscript{2} is the best candidate for the species which is phosphorylatable by ATP.

Mg\textsuperscript{2+} is needed for both phosphorylation reactions, and thus is thought to bind at least to the catalytic site. Guillain et al. (1982) and Moutin and Dupont (1991) suggested that Mg\textsuperscript{2+} could bind to one transport site. If this site were the second site, dissociation of the first Ca\textsuperscript{2+} ion would be impaired by the presence of a high concentration of Mg\textsuperscript{2+} (Fig. 8). Experimentally, we observed the opposite, Mg\textsuperscript{2+} has no effect on the dissociation of Ca\textsuperscript{2+}, indicating that it does not bind to the vacant Ca\textsuperscript{2+} binding site, although a slow binding of Mg\textsuperscript{2+} cannot be eliminated. Because there is evidence that the first Mg\textsuperscript{2+} binds to the catalytic site of EH\textsubscript{3}, Mg\textsuperscript{2+} is assumed to bind to the catalytic site in all of the species of the Ca\textsuperscript{2+} binding process, except for the Ca\textsuperscript{2+} saturated species, ECa\textsubscript{2}.

ATP is known to increase the apparent affinity of ATPase for Mg\textsuperscript{2+} in the absence of Ca\textsuperscript{2+}. We used this property, assuming it could be generalized to other nucleotides, especially to nonhydrolyzable analogs, in order to test whether Mg\textsuperscript{2+} would bind to ECa\textsubscript{2} in the presence of a nucleotide. ATP\textsubscript{8}S increased the apparent affinity for Mg\textsuperscript{2+}, as ATP does, and this made it possible for Mg\textsuperscript{2+} to bind to ECa\textsubscript{2}, as shown by the disappearance of the inhibition of Ca\textsuperscript{2+} binding by Mg\textsuperscript{2+} (Figs. 9 and 10).

![Scheme 2. A proposal for Ca\textsuperscript{2+}/H\textsuperscript{+} exchange at transport sites.](image-url)
The $\text{ECa}_2$ species binds $\text{Mg}^{2+}$ in the presence of nucleotide. This demonstrated that $\text{ECa}_2$ is the species which can be phosphorylated by ATP and reinforces the idea that $\text{Mg}^{2+}$ binds to the catalytic site during all of the $\text{Ca}^{2+}$ binding process. This result, together with the fact that $\text{EH}_3$ is the only species which can be phosphorylated by $\text{P}_i$, shows that Scheme 1 can be taken as a model for the $\text{Ca}^{2+}$ binding step in the ATPase cycle. In this regard, Fujimori and Jencks (1992) have recently shown that during $\text{Sr}^{2+}$ binding to the transport sites, three different chemical specificities can be observed; the $\text{Sr}^{2+}$-deprived species is only phosphorylatable by $\text{P}_i$, the species having one $\text{Sr}^{2+}$ bound does not react neither with $\text{P}_i$ nor with ATP, and the species having two $\text{Sr}^{2+}$ bound is only phosphorylatable by ATP. The results reported here agree with this sequence and bring more precision about the change in the chemical specificity. In the presence of $\text{Mg}^{2+}$, among the different species having free transport sites $\text{EH}_3$ is the only one which reacts with $\text{P}_i$ and $\text{ECa}_2$ is the only species which reacts with ATP. Thus, Scheme 1 emphasizes the progressive aspect of the change in the chemical reactivity. In addition, it points out the existence of two routes which can be taken by ATPase to go from a form which has no affinity for external $\text{Ca}^{2+}$, to a form which is phosphorylatable by ATP.

The fact that three $\text{H}^+$ are dissociated from ATPase during the binding of two $\text{Ca}^{2+}$ suggests that these three $\text{H}^+$ are at or close to the $\text{Ca}^{2+}$ sites and that they are involved in the proton countertransport which accompanies the uptake of two $\text{Ca}^{2+}$ per turnover (Yamaguchi and Kanazawa, 1984, 1985; Lévy et al., 1990). In Scheme 2, a mechanism for $\text{Ca}^{2+}/\text{H}^+$ exchange at the cytoplasmic side of the membrane is shown which accounts for our results.

In this model, 3 residues are assumed to be deprotonated during the $\text{Ca}^{2+}$ binding process. Dissociation of the three $\text{H}^+$ requires two steps which are tightly linked to the two $\text{Ca}^{2+}$ binding steps. Following the upper route, which applies at alkaline pH, the residues are deprotonated ($\text{pK}_7$ and 7.9) prior to the binding of $\text{Ca}^{2+}$ ($K_1 = K_2 = 0.1 \mu\text{M}$). Note that the $\text{Ca}^{2+}$ ions are piled up, as first proposed by Petithory and Jencks (1988a). Following the lower route, the first deprotonation ($\text{pK}_7$) allows the first $\text{Ca}^{2+}$ binding ($K_1' = 1.25 \mu\text{M}$). In the resulting $\text{EHCa}$ species, $\text{H}^+$ masks a negative charge and is not supposed to have any steric effect on $\text{Ca}^{2+}$ movements. The dissociation of the third $\text{H}^+$ ($\text{pK} 6.8$) is followed by the second $\text{Ca}^{2+}$ binding ($K_2 = 0.1 \mu\text{M}$). A symmetrical exchange is assumed to take place on the luminal side to ensure countertransport of $\text{H}^+$. This fits well with the result that $\text{EH}_3$ is the only species which can be phosphorylated by $\text{P}_i$. During the ATPase cycle, after the release of $\text{Ca}^{2+}$ in the lumen and dephosphorylation, ATPase would start a new cycle in a protonated form and exchange two $\text{Ca}^{2+}$ against three $\text{H}^+$ in each turnover.

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