Formation of a Stable Inactive Complex of the Sarcoplasmic Reticulum Calcium ATPase with Magnesium, Beryllium, and Fluoride*

(Received for publication, June 21, 1993, and in revised form, August 5, 1993)

Alexander J. Murphy‡ and Richard J. Coll
From the Department of Biochemistry, School of Dentistry, University of the Pacific, San Francisco, California 94115

Incubation of leaky or nonionic detergent-solubilized sarcoplasmic reticulum vesicles in solutions containing magnesium, beryllium, and fluoride caused a time-dependent complete inhibition of calcium ATPase activity. The inhibited state persisted through dialysis for 2 days versus EGTA and was reversed within minutes by the presence of 0.5 mM calcium. Calcium-independent ATPase activity was unaffected. Omission of magnesium or fluoride resulted in retention of activity, while omission of beryllium produced slower inactivation, as described previously (Murphy, A. J., and Coll, R. J. (1992) J. Biol. Chem. 267, 5229–5235). Incubation of nonleaky vesicles had a similar effect, although it occurred more than 10-fold more slowly, suggesting that a component, probably beryllium, must enter the vesicles for inhibition to occur. By contrast, inhibition of nonleaky vesicles by magnesium and fluoride developed less than 2-fold more slowly. Including calcium in the incubation mixtures resulted in partial protection, so that the time course of CaATPase activity leveled off at nonzero values (for example, at 0.1 mM calcium, the activity leveled off at 43% of control). This result is most simply accounted for by a model involving simultaneous binding of calcium and a form of fluoroberyllium to the CaATPase (e.g., Ca$_2$EMgBeF$_6$).

An essential step of relaxation of myocytes is active transport of calcium out of the cell’s cytosol into the lumen of SR$^+$ membranes. This ion pumping is performed by a calcium ATPase, with two calcium ions being transported for each ATP hydrolyzed (for recent reviews, see Inesi et al. (1992), Jencks (1989), and MacLennan (1990)). In common with other ion pumps, the enzyme undergoes ATP-driven phosphorylation (EP formation) during the transport cycle. This intermediate is hydrolyzed after calcium dissociates into the SR lumen and can be formed by incubation of the calcium-free enzyme with P$_i$ and magnesium.

Additional characterization of EP is central to detailed understanding of the mechanism of action of ion pumps. One approach has been study of complexes with putative P$_i$ analogs.

*This work is supported by National Institutes of Health Grant GM31083. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡To whom correspondence should be addressed. Tel.: 415-929-6678; Fax: 415-929-6654.

1 The abbreviations used are: SR, sarcoplasmic reticulum; Mops, 3-N-morpholino propane-1-sulfonic acid; EP, phosphorylated enzyme intermediate; C$_{12}$E$_6$, poly(oxyethylene)-9-lauryl ether; EMgF$_6$, or EMgBeF$_6$, inactive complexes of the CaATPase formed by incubation with magnesium and fluoride or with magnesium, beryllium, and fluoride, respectively; Vi, orthovanadate.

Orthovanadate, for example, has been found to interact with the phosphorylation site of the Na,K-ATPase (Cantley et al., 1978; Smith et al., 1980) and the SR CaATPase (O’Neal et al., 1979; Pick, 1982). More recently, we have shown that fluoride behaves similarly, with the slowly formed inhibited complex (EMgF$_6$) being much more stable and having lumen-facing calcium sites with about 10$^4$-fold lower affinity than those of the uninhibited enzyme (Murphy and Coll, 1992a, 1992b; Coll and Murphy, 1992). A comparison of these results with fluoride’s effect on the Na,K-ATPase revealed numerous parallels, with the effects of calcium being analogous to those of sodium and the effects of protons being analogous to those of potassium (Murphy and Hoover, 1992).

Another group of putative analogs of P$_i$ includes the fluoride complexes of aluminum acid beryllium (Chabre, 1990). These have been found to behave as effectors of wide variety of proteins that interact with P$_i$ and its derivatives. Among these proteins are adenylate cyclase (Sternweis and Gillman, 1982), transducin (Bigay et al., 1985), mitochondrial H$^+$-ATPase (Duquis et al., 1989), and myosin (Werber et al., 1992). Complex formation between fluoroaluminates and the SR CaATPase has been studied by measuring changes in the enzyme’s intrinsic fluorescence (Trouiller et al., 1992). The related fluoroberylliates might be expected to behave similarly with the potential advantage of greater simplicity because beryllium is a second-row element; whereas aluminum (by hybridization of 3d with 3s and 3p orbitals) can vary in coordination number from 4 to 6, beryllium (because utilizing 3d orbitals would be energetically highly unfavorable) is restricted to a coordination number of 4. We report here that incubation of the CaATPase of SR with magnesium, beryllium, and fluoride results in formation of a stable complex with significant similarities to and differences from the formation of EMgF$_6$. Some of these results have been reported in preliminary form (Murphy and Coll, 1993).

MATERIALS AND METHODS

Rabbit hind leg muscle was the source of SR vesicles (Eletr and Inesi, 1972). They were stored in 30% sucrose, 10 mM Mops, pH 7.0, on ice or at –80 °C after quick-freezing with liquid N$_2$. Solubilized SR ATPase was prepared as previously described (Coll and Murphy, 1984); stability of its ATPase activity was improved by routinely using C$_{12}$E$_6$ stock solutions that had been brought to 100 °C for at least 30 min and cooled before use (Coll and Murphy, 1986). Sigma was the source of Na$_2$ATP, NaADP, C$_{12}$E$_6$ (catalog no. 9641), and lactate dehydrogenase and pyruvate kinase (both type II). The ionophore A23187 was obtained from Calbiochem, and sodium fluoride (Puratronics) was from Alfa. The source of beryllium was an atomic absorption standard solution (Sigma-1000 μg of beryllium/ml in 1% HCl).

Calcium-dependent ATPase activities were measured by coupled assay (Anderson and Murphy, 1983) at 25 °C and pH 7.0 in a solution containing 0.3 mM NADH, 2 mM phosphoenolpyruvate, 80 mM KCl, 50 mM Mops, 5 mM MgCl$_2$, 0.3 mM CaCl$_2$, 1 mM EDTA, 3 mM MgATP, 0.5 μg/ml ionophore A23187, 50 μg/ml SR protein, and at least 4 and 16 units/ml pyruvate kinase and lactate dehydrogenase, respectively. The concentration of the product P$_i$ was calculated by dividing the absorb-
SR CaATPase Forms Stable Inactive Complex with Mg, Be, and F

Results

Effect of Beryllium, Fluoride, and Magnesium on the Calcium ATPase Activity of SR—Incubation of SR vesicles in solutions containing beryllium, fluoride, and magnesium resulted in time-dependent inhibition of calcium ATPase activity (Fig. 1). The process is adequately fitted by a first order function with a single rate constant ($k_{obs}$); under these conditions $k_{obs} = 0.4 \pm 0.1 \text{ min}^{-1}$. The requirement for Mg$^{2+}$ is illustrated by its omission resulting a very slow activity decrease ($k_{obs} < 0.003 \text{ min}^{-1}$, probably due to contaminating Mg$^{2+}$). Deletion of Mg$^{2+}$ from the incubation mixture resulted in essentially full retention of activity.

As reported previously (Murphy and Coll, 1992a), incubation with magnesium and fluoride causes time-dependent inhibition, so that deletion of beryllium from the incubation mixtures described in Fig. 1 reduced $k_{obs}$ by a factor of about 200 ($k_{obs} = 0.002 \text{ min}^{-1}$). This indicates that under the MgBeF conditions of Fig. 1 the competing reaction of forming a beryllium-free complex is not significant.

In addition to speeding the reaction, beryllium in the incubation mixture produced a complex with the CaATPase, which underwent partial reactivation during our usual assay conditions; to minimize this, we assayed at 25 °C and [Ca$^{2+}$]$_{free}$ = 10 μM instead of 37 °C and 100 μM.

Effect of ADP and Calcium on the Rate of Inhibition—Inhibitory complexes involving fluoride and aluminum or beryllium usually involve ADP or GDP (Chabre, 1990). If the SR calcium ATPase can form an additional complex including ADP (and perhaps calcium, as observed with fluoride and aluminum (Troullier et al., 1992)), then this second route to a stable complex would result in faster inhibition rates with ADP present. In fact, the presence of 100 μM ADP resulted in a 3-4-fold decrease in $k_{obs}$, while endogenous calcium caused a somewhat larger (5-6-fold) decrease (Fig. 1). With both ADP and calcium present, the inactivation rate was further decreased. These results imply that both ligands have a protective effect and that they do not promote the formation of an additional inactive complex such as Ca$_2$EADPBeF$_4$.

Effect of Inorganic Phosphate on the Rate of Inhibition—In addition to the three ions mentioned above, the inhibition reaction rate was markedly enhanced by the presence of the ionophore A23187: by omitting it, $k_{obs}$ was decreased by a factor of 30-40 (Fig. 2). To test for the possibility that the ionophore was facilitating the permeation and chelation by EGTA of protecting luminal calcium, we preincubated SR vesicles with ionophore and EGTA, removed the former with an anion-exchange column, and then incubated with Mg$^{2+}$, Be$^{2+}$, and F$^-$. The resulting inhibition rate was similar to vesicles not exposed to ionophore, implying that residual calcium was not responsible for the slow inhibition rate. The effect of this ionophore on $k_{obs}$ for the beryllium-free inhibition conditions was much smaller (Fig. 2); its inclusion caused $k_{obs}$ to increase by less than 2-fold.

Effect of Calcium on the Rate and Extent of Inhibition—We reported previously that inhibition by Mg$^{2+}$ and F$^-$ is completely prevented by the presence of calcium with a midpoint near 1 μM Ca$^{2+}$ (Murphy and Coll, 1992a). By contrast, with beryllium present calcium caused the inhibition to be rapid and

---

**Equation 1**

$$A = A_0 \exp \left[ - \left( k_1 + k_2 \right) t \right] + k_2 \left( k_1 + k_2 \right)$$
is usually characteristic of covalent bond formation, but more activation within minutes (Fig. 3). The presence of calcium in the millimolar range results in reactivation within minutes, while EGTA-containing solutions with little regain of activity, while there are significant differences. The two complexes are also similar in that they can be dialyzed for days at 4 °C versus a solution free of these ions and containing 1 mM EGTA. As shown in Fig. 4, reactivation within minutes was observed by incubation of the dialyzed vesicles with 0.5 mM calcium, showing that the inhibition process is reversible. Because the reactivation of dialyzed EMgBeF₄ produces very low concentrations of Be²⁺ and F⁻, essentially all of the original activity is regained. The figure also illustrates that omission of A23187 from the reactivation solution resulted in a decrease (approximately 5-fold) in the reactivation rate, consistent with calcium exerting its effect on the luminal side of the membrane.

**DISCUSSION**

We showed previously that incubation of the CaATPase of SR with magnesium and fluoride slowly brings about formation of a stable complex devoid of ATPase activity (EMgF₂) (Murphy and Coll, 1992a). We report here that inclusion of beryllium caused inhibition to develop much faster. By using a low fluoride concentration we can select for the beryllium-containing complex (EMgBeF₄) since the beryllium-free one forms at a rate that is second order in fluoride (Murphy and Coll, 1992a), and the inhibition process is reversible. Beryllium also has a protective effect, although as discussed below there are significant differences. The two complexes are also similar in that they can be dialyzed for days at 4 °C versus EGTA-containing solutions with little regain of activity, while the presence of calcium in the millimolar range results in reactivation within minutes (Fig. 4).

This degree of stability and relatively slow rate of formation is usually characteristic of covalent bond formation, but more likely what is happening is formation of noncovalent complexes involving slow, tight-binding enzyme inhibitors (Morrison and Walsh, 1988; Schloss, 1988). For reasons stated previously (Murphy and Coll, 1992a, 1992b; Coll and Murphy, 1992), the phosphorylation site of the ATPase is the most likely place where complex formation occurs, and it is plausible that the complexes are long-lived analogs of one or more intermediate states of the catalytic cycle. Additional characterization by such techniques as NMR has the potential for revealing details of the interaction between ligands and the phosphorylation site.

There are several interesting differences between the two complexes. While the presence of the calcium ionophore A23187 has a less than 2-fold effect on kobs for formation of EMgF₂, it has a 30–40-fold accelerating effect on EMgBeF₄ formation. This ionophore-dependent acceleration implies that access to the vesicle lumen by beryllium is an important component of the inhibition process. Another difference is in the calcium concentration dependence for reactivation. For EMgF₂ the midpoint is about 10 mM calcium (Murphy and Coll, 1992b), and for EMgBeF₄ it is below 1 mM calcium.² In contrast to the complete protection from EMgF₂ formation afforded by calcium in the micromolar range (Murphy and Coll, 1992a), when beryllium is included this divalent cation provided little protection in this range and only partial protection in the submillimolar range (Fig. 3). These results are most readily explained by postulating formation of a complex which includes both calcium and the inhibitory ions, e.g., Ca₂⁺EMgBeF₄ (Scheme 1).

Under conditions illustrated in Fig. 3, at time zero Ca₄⁺E > E, so that fast formation of inhibited enzyme upon adding fluoride and beryllium would occur via step 2. Quenching by addition of the incubation mixture to an assay mixture dilutes out both calcium and unbound fluoride and beryllium. The measured CaATPase activity reveals the percentage of enzyme that is not in a stable inactive form. To account for attainment of a partially active state, the extent of which depends on the concentrations of magnesium and calcium, steps 2 and 4 must be readily reversible.

These results resemble the effects of calcium and orthovanadate on the CaATPase activity and on the fluorescence of fluorescein-labeled SR, which showed that both ligands can bind to the enzyme simultaneously (Markus et al., 1986, 1989). The present study suggests a calcium affinity with a midpoint in the 0.1–1 mM range for EMgBeF₄; this is intermediate between that of EMgVi (0.8 μM; Markus et al. (1989)) and EMgF₂ (12 mM; Murphy and Coll (1992b)). By comparison, the calcium affinity

² R. J. Coll and A. J. Murphy, manuscript in preparation.

![Fig. 2. Effect of calcium on the time course of inhibition of SR CaATPase activity by magnesium, beryllium, and fluoride. Incubation conditions are described under "Materials and Methods," except that EGTA was omitted and 0.1 mM CaCl₂ (∅), 0.2 mM CaCl₂ (□), or 0.1 mM CaCl₂ and 15 mM MgCl₂ (△) was included.](image-url)

![Fig. 4. Time course of reactivation by calcium of SR CaATPase inhibited with magnesium, beryllium, and fluoride. Incubation conditions are described under "Materials and Methods." Reactivation was done in the absence (∅) or presence (△) of A23187 (25 μg/ml).](image-url)

![Scheme 1](image-url)
of EP is about 1 mM (Ikemoto, 1975; Prager et al., 1979; Pickart and Jencks, 1984).

As has been found for the reactivation of the vanadate (Medda and Hasselbach, 1983) and fluoride (Murphy and Coll, 1992b) complexes, the calcium-accelerated reactivation of EMgBeF₃ goes fastest when A₂₃₁₈₇ is included (Fig. 4), implying access to lumen-facing sites is involved. If these are the transport sites, then formation of these complexes simulates a central feature of ion pumping, namely simultaneous transformation of the orientation and ion affinity of transport sites. As a group, these complexes, with a range of calcium affinities, presumably have structures representing the range of intermediate states of the pumping cycle. Accordingly, they have the potential for serving as models or “snapshots” of the transient structures of the ion transporting pathway.

REFERENCES