Fluoride-inhibited Calcium ATPase of Sarcoplasmic Reticulum

MAGNESIUM AND FLUORIDE STOICHIOMETRY*

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The sarcoplasmic reticulum (SR) CaATPase is inactivated by fluoride in the presence of magnesium (Murphy, A. J., and Coll, R. J. (1992) J. Biol. Chem. 267, 5229–5235). The inactive complex is very stable and can be isolated free of other components by 48 h of dialysis at 4 °C (Murphy, A. J., and Coll, R. J. (1992) J. Biol. Chem. 267, 16990–16994). In this study, we used a fluoride-specific electrode to determine that the amount of tightly bound fluoride in the complex was 9.4 ± 2 nmol mg⁻¹ SR protein. The rate constant of inactivation was very similar to the rate constant of fluoride incorporation and varied directly as the square of the fluoride concentration. Luminal Ca⁺⁺ accelerated reactivation of the inhibited enzyme, and the rate constants of activity regain and fluoride release were very similar. Although required for inhibition, added magnesium did not accelerate reactivation.

Analysis for magnesium using antipyrylazo III of the inhibited enzyme showed 4.1 ± 0.4 nmol mg⁻¹ SR protein. As there is much evidence in the literature supporting of an estimate of calcium pumps equal to ~4–5 nmol mg⁻¹ SR protein, our results indicate that each inhibited enzyme contains two tightly bound fluorides and one tightly bound magnesium.

The major reservoir of calcium in muscle cells is the sarcoplasmic reticulum. Release of calcium from the SR causes contraction; subsequent active transport of calcium into the lumen of the SR causes relaxation. Embedded in the SR membrane is the CaATPase that performs this transport (for recent reviews, see Inesi et al. (1992), Jeneck (1989), and MacLennan (1990)). The process of transport necessitates the conversion of cytosol-facing high affinity calcium-binding sites into lumen-facing ones of low affinity. This process is coupled to ATP hydrolysis, which involves transfer of the γ-phosphoryl of ATP to the enzyme to form EP; after release of calcium to the lumen, EP hydrolyzes.

Enzymes known to not release reactive (transition-state) intermediates prior to release of product; certain slow tight-binding inhibitors are thought to mimic these intermediates (Morrison and Walsh, 1988). We recently reported that fluoride is a slow tight-binding inhibitor of the SR CaATPase (Murphy and Coll, 1992a). This inhibition requires magnesium and is prevented by cytosolic calcium, P₄, and orthovanadate. The rate constant of inhibition follows a second-order dependence on [F⁻]. It was later found that the inhibited enzyme possesses its calcium-binding sites in a low affinity, lumen-facing orientation (Murphy and Coll, 1992b). This report further characterizes this inhibited enzyme with regard to the stoichiometry of fluoride and magnesium and to the relative rates of fluoride binding and enzyme inhibition.

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 N₂. Fluoride-inhibited SR vesicles were prepared (Murphy and Coll, 1992a) and stored at −80 °C after dialysis. Na,ATP, NaADP, antipyrylazo III, pyruvate kinase and lactate dehydrogenase (both type II), and calcium ionophore A23187 were obtained from Sigma; NaF (puratronic) was from Alfa Products. Calcium-dependent ATPase activity was measured at 37 °C and pH 7.0 as described previously (Anderson and Murphy, 1983; Murphy and Coll, 1992a). Control specific activities were in the range of 15–20 nmol min⁻¹ mg⁻¹.

Fluoride Incorporation into and Release from SR CaATPase—To measure fluoride concentrations, we used a fluoride-specific electrode (Orion 966900) according to the manufacturer’s directions. Unless otherwise noted, all experiments with the electrode were in 2 ml of 50 mM Mops, 1 mM EGTA, pH 7.0; steady millivolt readings were obtained within 5 min. To obtain the stoichiometry of fluoride incorporation, samples of inhibited SR (2–8 mg of protein) that had been separated from free F⁻ and Mg⁺⁺ by dialysis (Murphy and Coll, 1992a) were equilibrated with the electrode to obtain a reference voltage; subsequently, the enzyme was denatured (final concentration of 2% sodium dodecyl sulfate or 10 min of exposure to 100 °C) to release bound fluoride, and after cooling, the voltage was measured again.

In some experiments, the inhibited SR vesicles were embedded in filters (Millipore HAWP, 0.45 μm) so that the medium surrounding the enzyme could be rapidly changed, e.g. to wash unbound fluoride. In these cases, 2.5 mg of inhibited SR was diluted with 5 ml of buffer (50 mM Mops, 1 mM EGTA, pH 7.0) and injected through four stacked (buffer-prewashed) 25-mm filters in a plastic Swinnex holder. The embedded SR was then washed with two 5-ml portions of buffer; after each application of solution through the filters, the dead volume of the holder was flushed through with air. The absorbance (280 nm) of solutions passed through the filters was monitored to ensure that the capacity of the filters for the SR had not been exceeded. Tightly bound fluoride was released from the embedded SR by boiling the filters in 50 mM Mops, 1 mM EGTA for 10 min.

Fluoride release from diazylated inhibited SR was initiated by the addition of calcium to vesicles that had been made leaky by inclusion of ionophore A23187 (one-twentieth the weight of SR protein). At given time points, aliquots were removed and quenched with excess EGTA, and free fluoride was measured. The time course of fluoride incorporation into the SR was followed in a solution containing 8–12 mg/ml SR, 30% (v/v) sucrose, 10 mM Mops, pH 7.0, 1 mM EGTA, 5 mM MgCl₂, and 1 or 2 mM NaF at 39 °C. At appropriate time intervals, 0.5-ml aliquots of the inactivation mixture were diluted with 10 volumes of ice-cold 50 mM Mops, 1 mM EGTA; quick-frozen with liquid N₂; and stored at −80 °C. The subsequently thawed samples were quickly diluted, and the SR was absorbed onto filters and treated as described above to determine bound fluoride.

Determination of Bound Magnesium—All the solutions used in the...
analysis of Mg\(^2+\) were passed through Bio-Rex ion-exchange membranes (Chelex) from Bio-Rad to remove contaminating multivalent cations. Inhibited and dialyzed SR CaATPase (50–70 mg) was placed in plastic tubes and pelleted. To the pellets was added 2 ml of Chelex-treated 0.1 M triethanolamine/Mes, pH 8.0, and the capped tubes were placed in a boiling water bath for 1 h with periodic trituration. The supernatants of the recenterfuged pellets were further clarified in a microcentrifuge. To an aliquot of supernatant corresponding to 5 mg of SR was added 90 \(\mu\)M Chelex-treated antipyrilazo III, and a difference spectrum versus the dye and no SR was recorded from 460 to 670 nm. The absorbance difference between a peak at 505 nm and a trough at 575 nm was taken as proportional to magnesium; an addition of 25 nmol of Mg\(^2+\) was then traced as an internal standard. Addition of 1 mM EDTA to each cuvette gave a difference spectrum corresponding to zero metal concentration. Sometimes the pellets had to be further extracted with as much as 4 × 1-ml Chelex-treated buffer to remove all the divalent cation. A control enzyme treated exactly the same as the inhibited one (except fluoride was omitted) was also analyzed and gave negligible signal change. To discriminate between Mg\(^2+\) and other metals (e.g. Ca\(^2+\)), after the initial trace was taken, an aliquot (250 \(\mu\)M) of either EGTA or EDTA was added. For the case of Mg\(^2+\), addition of EGTA caused negligible signal change, whereas EDTA brought the signal to zero. With Ca\(^2+\), either chelator removed the difference spectrum. For determination of Mg\(^2+\), the sample absorbance difference was either compared with the internal standard or was read from a linear standard curve prepared using known additions of MgCl\(_2\) to the control enzyme. Both methods gave similar estimates.

Data Analysis—Data were fit using single exponential functions as described previously (Murphy and Coll, 1992a, 1992b).

RESULTS

Comparison of Time Courses of Inhibition and Reactivation of CaATPase Activity with Fluoride Binding—We wished to study the kinetics of both fluoride inhibition and the subsequent reactivation of the inhibited calcium pump to gain insight into the mechanism. In particular, it was of interest to determine if these processes are multistep, including whether the fully inactive/active enzyme is preceded by a partially active form. Fluoride incorporation was followed with an ion-specific electrode, and the rate of formation of the tightly bound complex was compared with the rate of activity loss. Fig. 1 shows the data collected for enzyme inhibition with 1 and 2 mM fluoride. Both processes, fluoride binding and activity loss, are well described by single exponential functions. As can be seen in Table I, activity loss and fluoride incorporation yield \(k_{obs}\) values that are not significantly different. When the fluoride concentration is doubled, the rate constants more than double, which agrees with the previously described (Murphy and Coll, 1992a) second-order nature of fluoride inhibition.

![Fig. 1. Time course of fluoride incorporation into SR vesicles and parallel CaATPase measurements. Experiments were done as described under "Materials and Methods." Fluoride incorporation (• and △) and CaATPase activity (○ and □) were measured in incubation mixtures containing 1 or 2 mM NaF (○ and □, respectively).](image)

![Fig. 2. Time course of fluoride release from inhibited SR vesicles and parallel CaATPase measurements. Experiments were done as described under "Materials and Methods." Fluoride release (○ and ▲) and CaATPase activity (□ and △) were measured in incubation mixtures containing 1 or 2 mM CaCl\(_2\) and 30% sucrose.](image)

![Table I: Stoichiometry and rate constants for the inhibition and reactivation of the SR CaATPase by fluoride](image)

Analysis-Data were fit using single exponential functions as described under "Materials and Methods." Fluoride incorporation was followed in incubation mixtures containing 1 and 2 mM fluoride. Both processes, fluoride activity loss. Fig. 1 shows the data collected for enzyme inhibition and reactivation (Murphy and Coll, 1992b). We used this luminal Ca\(^{2+}\) to vary the rate of activity regained and to compare it with the amount of remaining tightly bound fluoride. Fig. 2 shows representative kinetics of fluoride interacting with SR and the parallel calcium pump activity. The time courses of fluoride dissociation and CaATPase reactivation are well described by monophasic first-order processes. Within experimental error, the rate constants for change in enzymatic activity and fluoride binding were the same (Table I). The concentration of reactivating calcium was varied beyond that shown in Fig. 2, giving a range of reaction velocities; in each condition, the rates of fluoride release and activity regain were similar. In Fig. 1, free fluoride was removed rapidly (filtration); and in Fig. 2, 48-h dialysis was used. The reasonable agreement in stoichiometry from both methods argues that the SR tightly binds little or no fluoride other.
than that which is released concomitant with activity regain. In agreement with this conclusion, when inhibited SR CaATPase was induced by calcium to full activity regain (Fig. 2), subsequent denaturation of the SR resulted in no further release of fluoride.

Effect of Magnesium on Reactivation of Fluoride-inhibited CaATPase and Stoichiometry of Tightly Bound Magnesium—The extensively dialyzed inhibited enzyme was studied for the effect of Mg\(^{2+}\) on its ability to reactivate. When the enzyme was reactivated by luminal calcium (Murphy and Coll, 1992b), it was found that added millimolar magnesium caused retardation of activity regain. This lack of Mg\(^{2+}\) catalysis of reactivation is also seen with the calcium-free case and suggests a stoichiometric role for Mg\(^{2+}\).

The dialyzed inhibited pump was therefore analyzed for bound metal with the dye antipyrylazo III. Upon denaturation, the inhibited enzyme released a metal that, when added to the dye, gave a difference spectrum identical to magnesium. Small amounts of added EDTA effectively competed with the dye for the metal, whereas small amounts of EGTA did not. These results lead us to conclude that the inhibited complex contains 4.1 ± 0.4 nmol mg\(^{-1}\) tightly bound magnesium.

**DISCUSSION**

Our measurements of fluoride bound to SR vesicles suggest that binding of two fluorides/CaATPase is sufficient for inhibition. Whereas some investigators favor an ATPase content of 7-8 nmol/mg of SR protein, stoichiometry measurements based on enzyme phosphorylation by ATP (Froehlich and Taylor, 1975) or P, (Inesi et al., 1984), reaction with fluorescein isothiocyanate (Andersen et al., 1982; Mitchinson et al., 1982; Highsmith and Murphy, 1984; Murphy, 1988), binding of trinitrophenyl nucleotides (Dupont et al., 1982; Coll and Murphy, 1986), and purification of the ATPase (Coll and Murphy, 1984) give values in the range of 4-5 nmol/mg. Our mean value of 9.4 nmol of F\(^{-}\)/mg therefore makes two fluorides per ATPase the most likely stoichiometry. This is consistent with inhibition kinetics that are second-order with respect to fluoride (Murphy and Coll, 1992a).

As Table I shows, the rate of inhibition exhibits a squared dependence on [F\(^{-}\)]. Within the error of our measurements, enzyme activity loss is concomitant with fluoride binding, and we cannot resolve any differences in the rates of binding of the two fluorides. The apparent dissociation constant for luminal calcium in reactivation is ~12 mM (Murphy and Coll, 1992b), so that the reactivations shown in Fig. 2 with 1 and 2 mM Ca\(^{2+}\) are in the linear regime of the binding curve. Table I shows that, upon doubling of luminal calcium, the rates roughly double; again there is no significant difference between the rate constants of fluoride release and activity regain. These parallels in both the fluoride inhibition and reactivation reactions rule out complex mechanisms in which inhibition and its reversal are slower or faster than binding and dissociation of two fluorides.

The inhibition reaction requires magnesium, but because reactivation is not accelerated by Mg\(^{2+}\), the principle of microscopic reversibility requires that the metal is not acting only catalytically. This stoichiometric role implies that the inhibited enzyme contains tightly bound magnesium. We tested this and determined that the amount of tightly bound Mg\(^{2+}\) is 4.1 ± 0.4 nmol mg\(^{-1}\), which means there is one metal ion per inhibited pump. The dissociation constant for Mg\(^{2+}\) in the inhibition reaction is 4 mM (Murphy and Coll, 1992a), which is in the range of K\(_{i}\) values reported for Mg\(^{2+}\) associated with the interaction of the ATPase with P, (Punzengruber et al., 1978; Inesi et al., 1984) and ATP analogs (Pang and Briggs, 1977, Murphy, 1990), with intrinsic fluorescence changes (Guillain et al., 1982), and with fluorescence energy transfer (Highsmith, 1984).

Based on these results, the inhibited pump may be designated MgEF\(_{2}\). Its calcium-binding sites are in a low affinity, lumen-facing form. Given that the fluoride inhibition reaction requires magnesium and is prevented by P, or vanadate and that the resulting complex does not form EF\(_{2}\) from P, and contains tightly bound magnesium, we suggest that fluoride binds at the phosphorylation site. This implies that the inhibited enzyme is a model for the reaction intermediate commonly referred to as E\(_{2}P\). For the purpose of discussion, we define E\(_{2}P\) as the phosphorylated enzyme that has its ion-transporting sites facing the lumen. During turnover under standard reaction conditions, only low levels of E\(_{2}P\) are present since its rate of hydrolysis is faster than the rate of its formation (Froehlich and Taylor, 1975; Takasawa and Tonomura, 1978). E\(_{2}P\) can be formed from P, in rapid equilibrium (de Meis and Inesi, 1982), but there is no evidence for the existence of ligand-free E\(_{2}\) (Murphy and Jencks, 1991).

The results presented in this report and in earlier work (Murphy and Coll, 1992a, 1992b) indicate that fluoride binding causes conversion of the polarity of calcium-binding sites in a fashion similar to that caused by enzyme phosphorylation. The similarity extends to lowering of the sites’ calcium affinity, indicating that the interaction energies between fluoride and calcium and between phosphate and calcium are in the same range (7-10 kcal mol\(^{-1}\)) (Pickart and Jencks, 1984; Murphy and Coll, 1992b). This mutual destabilization between sites, an essential feature of energy-transducing systems (Jencks, 1980), appears to be simulated well by fluoride binding. Therefore, MgEF\(_{2}\) appears to provide a stable model with many similarities to the normally elusive reaction intermediate, E\(_{2}P\).

Another similarity between the fluoride-inhibited enzyme and E\(_{2}P\) is the necessity for a divalent cation. Formation of E\(_{2}P\) from P, requires Mg\(^{2+}\) and the absence of cytosolic calcium; formation of MgEF\(_{2}\) from P, has the same requirements (Murphy and Coll, 1992a). There is evidence that the phosphorylated enzyme contains tightly bound Mg\(^{2+}\). This evidence includes studies on EF decomposition to P; and ATP and the lack of an effect of added EDTA on these reactions (Garrabrant et al., 1976; Dupont, 1980; Takakawa and Ranaizawa, 1982). In addition, it has been reported that metal-free ADP is the true substrate for the back reaction, implying that Mg\(^{2+}\) is still bound to EF (Yamada and Ikemoto, 1980). Also, when "CaATP is used as the substrate, one additional tightly bound calcium above the two transported calciums is observed, and it stays bound to E\(_{2}P\) (Shigekawa et al., 1983).

The characteristics of fluoride inhibition of the SR CaATPase\(^{2}\) that have been found so far are as follows. The inhibition reaction exhibits a second-order dependence on [F\(^{-}\)] that shows no evidence of saturation even at very high concentrations. There is a strict requirement for Mg\(^{2+}\) and the absence of calcium bound to the high affinity cytosolic transport sites. P, and vanadate provide complete protection in a competitive manner, whereas nucleotides provide partial antagonism to the inhibition. Despite the protective effects of individual ligands, fluoride surprisingly is able to inhibit the pump undergoing turnover. This implies that, in addition to the free enzyme, an intermediate in the pumping cycle is also susceptible to inhibition by F\(^{-}\). The pH dependence of the calcium-induced reactivation of the isolated inhibited pump has a pK\(_{a}\) >8, whereas inhibition shows a pK\(_{a}\) of 7.2.

\(^{2}\)These characteristics are similar to those reported for fluoride inhibition of the related Na,K-ATPase (Murphy and Hoover, 1992).
Considering that Ca\(^{2+}\)-induced reactivation occurs at luminal sites on the CaATPase, this suggests that, as proposed earlier (Chiesi and Inesi, 1980; Levy et al., 1990), protons may be counterported during normal turnover. The reactivity of sulfhydryl groups to 5,5'-dithiobis(nitrobenzoic acid) with the inhibited enzyme is similar to the pattern seen with the enzyme undergoing turnover rather than resting. Considering the results as a whole, Mg\(\text{EF}_2\) seems to be a stable model for E\(\text{ZP}\).

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