Biochimica et Biophysica Acta 854 (1986) 257–264
Elsevier

BBA 72883

Fluorometric titration of the sarcoplasmic reticulum adenosinetriphosphatase calcium sites in the presence of vanadate

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(Received July 1st, 1985)

Key words: Sarcoplasmic reticulum; Ca^{2+}-ATPase; Ca^{2+} binding site; Vanadate; Fluorescence; (Rabbit muscle)

Titration of the specific calcium binding sites of sarcoplasmic reticulum ATPase was carried out by measurements of intrinsic fluorescence in the absence and in the presence of vanadate. The previous finding that vanadate binding to the enzyme inhibits high-affinity calcium binding was confirmed. In addition, taking advantage of the slow kinetics of vanadate association and dissociation from the enzyme, we were able to titrate the fraction of sites remaining in the high affinity state in the presence of non-saturating vanadate. These sites were demonstrated to retain the characteristics displayed by the high-affinity sites in the absence of vanadate, and yielded information consistent with a competitive inhibition between vanadate and calcium. Reversal of the vanadate effect and reconversion of the binding sites to the high-affinity state was demonstrated by adding appropriate calcium concentrations to the enzyme-vanadate complex, and showing the appearance of the intrinsic fluorescence signal which is indicative of calcium occupancy of the sites in the high-affinity state. Partial or total reversal of the vanadate effect was obtained with very slow kinetics following addition of micromolar calcium or, at a somewhat faster rate, following addition of millimolar calcium. The latter experiments yielded titration of the binding sites in the low-affinity state, with a dissociation constant of approx. 2 mM at neutral pH and 10 mM Mg^{2+}. The time course of the fluorescence rise following addition of calcium in the presence of vanadate was more rapid in 'leaky' than in native sarcoplasmic reticulum vesicles, suggesting an intravesicular orientation of the low-affinity calcium sites involved in the reversal of the vanadate effect. Our observations provide experimental support for the postulated mechanism of high- and low-affinity interconversion of the ATPase calcium binding sites, and its dependence on the occupancy of the phosphorylation site by vanadate.

Introduction

It is commonly believed that the sarcoplasmic reticulum ATPase has two interconverting classes of calcium binding sites which are involved in enzyme regulation and calcium transport. When the enzyme activated by calcium bound to high-affinity sites is phosphorylated with ATP, the affinity of these sites is lowered and the orientation is changed to sustain vectorial transport of calcium against a concentration gradient. Following hydrolytic cleavage of the phosphoenzyme then, the calcium sites regain their high affinity and their original orientation, and a subsequent cycle occurs [1–7].

While the high affinity calcium sites have been

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Abbreviations: Mops, 4-morpholinepropanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; $C_{12}E_8$, dodecyl octaoxyethyleneglycol monoether.

titrated quite well by radioactive tracer and optical method in the absence of ATP [8–14], titration of the calcium sites in the low-affinity state is quite difficult, owing to the coexistence of numerous low-affinity sites of non-specific nature which also bind radioactive tracer. It is assumed that the low-affinity state titrates in the millimolar range, because 'back inhibition' of ATPase activity [15], calcium-dependent effects on spin-labeled phosphoenzyme [16], and formation of ATP upon addition of ADP to the enzyme phosphorylated with inorganic phosphate [17,18], are obtained upon addition of mM calcium. However, a good titration curve of the calcium sites in the low-affinity state was never obtained.

It is now well known that vanadate inhibits the sarcoplasmic reticulum ATPase, likely due to formation of a pentacoordinate complex at the phosphorylation site of the sarcoplasmic reticulum ATPase, exhibiting characteristics of a stereo analogue of phosphate at the site [19–21]. Vanadate binding to sarcoplasmic reticulum ATPase also reduces the affinity of the enzyme for calcium [22–24]. We have then carried out a series of experiments with the aim of titrating the low-affinity sites in the vanadate-enzyme complex. While the phosphoenzyme formed with inorganic phosphate is quite unstable and is transformed rapidly to the high-affinity state upon addition of calcium [25], we found that the vanadate-enzyme complex is sufficiently stable and the interconversion of the two states sufficiently slow to allow differential titration of low- and high-affinity calcium sites by measurements of intrinsic protein fluorescence.

Materials and Methods

Native sarcoplasmic reticulum vesicles were prepared from rabbit hind leg white muscle as previously described [26].

Sarcoplasmic reticulum ATPase was prepared as described by Meissner et al. [10], and referred to, heretofore, as 'leaky' vesicles.

Free Ca^{2+} concentrations were estimated from total Ca^{2+} and EGTA by computation [27] using the binding constant for Ca · EGTA given by Schwartzenbach et al. [28], the pK values for EGTA given by Blinks et al. [29] and taking into account pH and Mg^{2+} concentrations.

Protein concentration was estimated by the Lowry method [30] using bovine serum albumin as standard.

Measurements of fluorescence intensity were carried out with an Aminco-Bowman spectrofluorometer equipped with thermostated cell holder at 25°C and cuvette stirrer. Excitation wavelength was 290 nm and the emission intensity was measured at 330 nm. The medium contained 50 mM Mops (pH 6.8)/80 mM KCl/10 mM $MgCl_2$/1 mM EGTA/0.1 mg sarcoplasmic reticulum per ml. Vanadate solutions were prepared by dissolving NH_4VO_3 in 1 M NaOH and adjusting to pH 8.0. The stock solution of vanadate was 1 mM. Both the alkaline pH and the low concentration used prevent formation of polyvanadate species. The stock solution remained colorless at all times. Vanadate was added in micromolar concentrations to sarcoplasmic reticulum at least 10 min before the fluorescence measurements. Titrations were performed by adding calcium to yield different free Ca^{2+} concentrations, and using fresh samples for each measurement.

Fluorescence kinetic transients were measured at 25°C with a Dionex D-137 stopped-flow spectrofluorometer equipped with a 75 W mercury-doped xenon lamp. The excitation wavelength was selected at 290 nm with a monochromator and the emitted light was passed through a 0-54 Corning cut-off filter. The digitized signals were collected with a North Star Horizon microcomputer for storage and analysis.

Results

An example of the enhancement of intrinsic fluorescence produced by high-affinity calcium binding to native sarcoplasmic reticulum ATPase is shown in Fig. 1. When sarcoplasmic reticulum vesicles are preincubated in the presence of 1 mM EGTA, addition of 0.9 mM calcium to yield 9 μM free calcium induces an increase in the protein tryptophan fluorescence (Fig. 1a) as originally described by Dupont [11]. We have previously demonstrated that 9 μM calcium saturates the high-affinity sites of sarcoplasmic reticulum ATPase [14].

If the enzyme is preincubated with 1 mM EGTA and 1 μM vanadate (Fig. 1b), the fluorescence enhancement after calcium addition correspond to

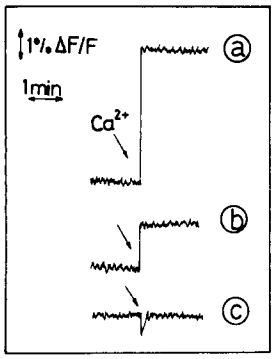


Fig. 1. Micromolar Ca^{2+}-induced intrinsic fluorescence changes at equilibrium in sarcoplasmic reticulum vesicles. The excitation wavelength was 290 nm and the emission 330 nm. Temperature, 25°C. Reaction mixture: 50 mM Mops (pH 6.8)/80 mM KCl/10 mM $MgCl_2$/1 mM EGTA/0.1 mg sarcoplasmic reticulum per ml. (a) The fluorescence transition was evoked by adding 0.9 mM Ca^{2+} to give 9 μM free concentration. (b) Sarcoplasmic reticulum ATPase was preincubated in the presence of 1 μM vanadate for 30 min before the addition of 0.9 mM Ca^{2+}. (c) The reaction mixture included 10 μM vanadate and after preincubation, 0.9 mM Ca^{2+} was added.

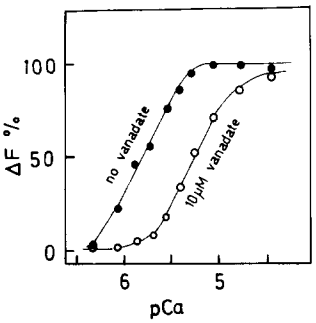


Fig. 2. Titration of the high-affinity calcium sites of leaky vesicles. Reaction mixture and other experimental conditions as described under Materials and Methods. Fluorescence changes were measured 10 min after addition of Ca^{2+}.

35% of that obtained in the absence of vanadate. When the vanadate concentration in the preincubation medium is 10 μM, no measurable fluorescence enhancement is observed (Fig. 1c) soon after addition of calcium, although a slow fluorescence rise may in fact occur on a longer time-span (minutes). In this case, the fluorescence rise is much slower than in the absence of vanadate, indicating that this signal is obtained only after dissociation of vanadate from the enzyme, which was previously found to occur at slow rates [21–23].

It is then possible to measure the Ca^{2+} concentration dependence of the fluorescence changes at equilibrium, in the absence and in the presence of vanadate (Fig. 2). These fluorescence measurements are similar to the equilibrium binding isotherms obtained following prolonged incubation of native sarcoplasmic reticulum vesicles with calcium and its radioactive tracer [23,24], and are consistent with a competitive inhibition of vanadate on calcium binding.

Taking advantage of the very slow effect of micromolar calcium on the enzyme-vanadate complex of 'native' vesicles, we were able to titrate the calcium sites remaining in the high-affinity state in the presence of non-saturating vanadate. These experiments were performed by preincubation of sarcoplasmic reticulum vesicles in the presence of EGTA (to remove calcium from the high-affinity sites) and various concentrations of vanadate. As the vanadate concentration in the medium was increased, the fluorescence effect of calcium (in the μM range) decreased. Therefore, we assume that vanadate stabilizes the enzyme in the low-affinity state, and the fluorescence changes produced by calcium in the μM range represent in each case the fraction of enzyme remaining in the high-affinity state.

It was previously demonstrated that the equilibrium isotherm of high-affinity calcium binding to sarcoplasmic reticulum ATPase is consistent with a cooperative mechanism [12]. The shape of the titration curves obtained in the presence of non-saturating vanadate shows that the cooperative mechanism is maintained for the fraction of high affinity sites which is not affected by vanadate (Fig. 3A). In all cases, Hill plots yield slopes of approx. 2 (Fig. 3B). Additional support is provided by the Scatchard plots showing parabolic curves as predicted for positive cooperativity (Fig. 3B). In the presence of low vanadate concentrations, the residual high affinity sites are half-saturated (K_s) at a pCa (5.85) which is in the same range reported in the absence of vanadate [14]. This confirms that vanadate decreases specifically the population of high-affinity calcium sites involved in enzyme activation.

The inhibitory effect of vanadate on the

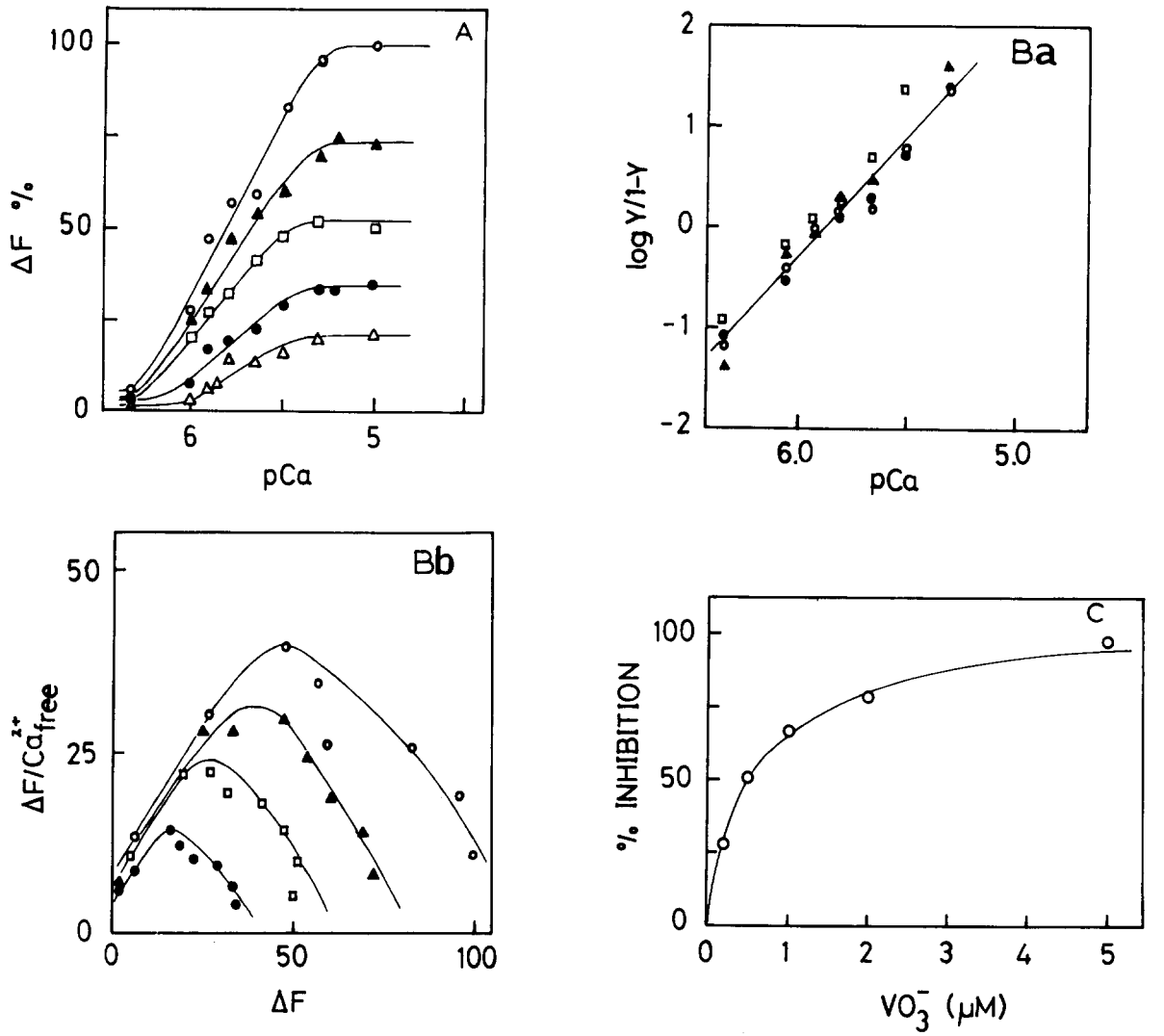


Fig. 3. (A) Titration of the high-affinity calcium sites in the presence of different amounts of vanadate. Reaction mixture and other experimental conditions as described under Materials and Methods. Fluorescence changes were measured after preincubation of sarcoplasmic reticulum vesicles for 10 min with vanadate, by adding Ca^{2+} to yield the required p*Ca*. Vanadate concentrations: none (○), 0.2 μM (▲), 0.5 μM (□), 1 μM (■) and 2 μM (△). (B) Hill (a) and Scatchard (b) plots of the high-affinity calcium sites in the presence of vanadate. The experimental points are taken from Fig. 3A. The vanadate concentrations plotted are: none (○), 0.2 μM (▲), 0.5 μM (□) and 1 μM (●). $Y = \Delta F/\Delta F_{max}$. (C) Percentage of the fluorescence signal inhibition as a function of the vanadate concentration. From the plotted data can be estimated a dissociation constant for the ATPase vanadate complex of 0.5 μM.

calcium-induced fluorescence changes, follows a hyperbolic pattern (Fig. 3C). The resulting curve yields the affinity of vanadate for the enzyme ($K_d \approx 0.5$ μM).

In an other set of experiments, we measured the kinetics of the fluorescence rise upon addition of calcium in a concentration sufficient to saturate the sites remaining in the high affinity state in the presence of non-saturating concentrations of vanadate. This was accomplished by preincubating sarcoplasmic reticulum vesicles in the presence of EGTA with different concentrations of vanadate in one syringe, and then initiating the reaction in a stopped-flow apparatus by adding calcium from the other syringe to provide a final free calcium concentration of 32.4 μM (Fig. 4).

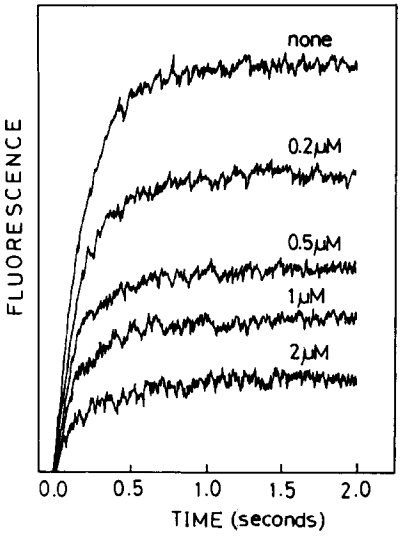


Fig. 4. Initial rates of calcium binding to the high-affinity sites of native sarcoplasmic reticulum vesicles, in the presence of vanadate. The reaction medium was: 50 mM Mops (pH 6.8)/80 mM KCl/10 mM $MgCl_2$. Reactions were initiated with the aid of a Dionex stopped-flow apparatus. One syringe contained sarcoplasmic reticulum vesicles (0.2 mg/ml) preincubated with 2 mM EGTA and vanadate. The other syringe delivered an equal volume of the same medium containing 2 mM Ca^{2+}. Vanadate concentrations after mixing are indicated in the figure. The time constant was 10 ms. Each trace is the average of three or more experiments.

In spite of the effect of vanadate on the final level of fluorescence, the observed rate constant, k_{obs}, for the calcium-induced fluorescence changes was found to be 4.7 s^{-1} at different vanadate concentrations, as well as in the absence of vanadate. Therefore, k_{obs} is independent of vanadate. Furthermore, saturation of the fraction of sites remaining in the high-affinity state does not influence the sites kept in the low-affinity state by vanadate within the time-scale of the rapid kinetic measurements.

A useful feature of the calcium effect on the intrinsic fluorescence of sarcoplasmic reticulum ATPase is its specificity for occupancy of calcium transport sites. In fact, in the absence of vanadate, a fluorescence signal is obtained only by titration of high-affinity sites with calcium in the micromolar concentration range. No further increase of intrinsic fluorescence is observed when the calcium concentration is raised up to millimolar range. On the contrary, in the presence of vanadate con-

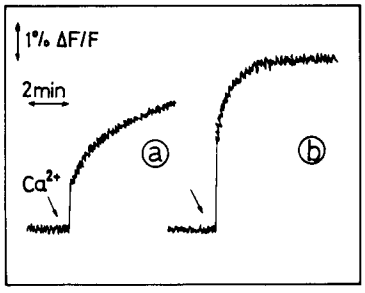


Fig. 5. Intrinsic fluorescence changes induced by millimolar calcium in native sarcoplasmic reticulum vesicles preincubated with 10 μM vanadate. Experimental conditions and reaction mixture as described in Fig. 1. The reaction was initiated by adding 2 mM Ca^{2+} (a) or 7.5 mM Ca^{2+} (b).

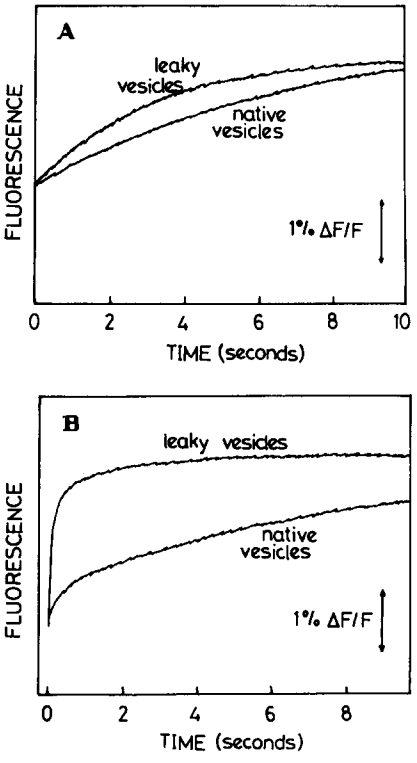


Fig. 6. Fluorescence rise induced by high calcium in 'native' and 'leaky' sarcoplasmic reticulum vesicles preincubated with vanadate. The reaction mixture contained: 50 mM Mops (pH 6.8)/80 mM KCl/10 mM $MgCl_2$/1 mM EGTA/0.1 mg protein per ml/10 μM vanadate. The fluorescence changes were induced by the addition of Ca^{2+} to obtain 1 mM (A) or 5 mM (B) free Ca^{2+}. 'Leaky' vesicles were obtained by the addition of $C_{12}E_8$ to a final concentration of 0.1 mM in the reaction mixture containing 0.1 mg sarcoplasmic reticulum protein per ml. This low concentration of detergent produces 'leakiness' without solubilizing the vesicles.

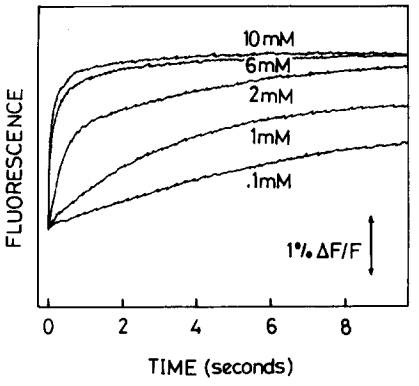


Fig. 7. Fluorescence rise induced by addition of calcium to 'leaky' sarcoplasmic reticulum vesicles preincubated with vanadate. Experimental conditions as for Fig. 6. The final concentrations of free Ca^{2+} are indicated in the figure.

centrations producing inhibition of high-affinity calcium binding, addition of millimolar calcium produces a fluorescence rise which finally reaches the full effect produced by micromolar calcium in the absence of vanadate (Fig. 5). This observation is consistent with interconversion of the calcium binding sites, from the high- to the low-affinity state as a consequence of vanadate addition, and back from the low- to the high-affinity state following addition of millimolar calcium (i.e., calcium occupancy of the sites kept by vanadate in the low-affinity state).

It is of interest that the rate of fluorescence rise is much faster when millimolar Ca^{2+} is added to 'leaky' than to 'native' vesicles (Fig. 6A and B). This suggests that the calcium sites change orientation in addition to undergoing a reduction in affinity as a consequence of vanadate binding to the ATPase.

Inspection of the kinetics of intrinsic fluorescence rise following addition of calcium in the millimolar range to 'leaky' vesicles preincubated with saturating (10 μM) vanadate, reveals a relatively fast and a slow component. The size of the fast component increases as the calcium concentration is raised (Fig. 7). A plot of the extent of the fast fluorescence rise as a function of Ca^{2+} concentration (not shown), yields a curve with half maximal saturation at approx. 2 mM Ca^{2+}. Addition of 10 mM calcium induces the entire fluorescence rise at the faster rate.

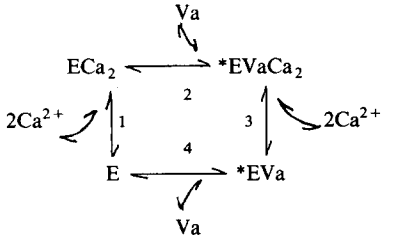
Discussion

The active transport of calcium sustained by the sarcoplasmic reticulum ATPase is attributed [1–7] to interconversion of the ATPase calcium binding sites into two states with K_s of approx. 1 μM and 1 mM. In the presence of calcium, conversion to the low-affinity state is produced by enzyme phosphorylation by ATP (or another suitable substrate), thereby permitting release of bound calcium against a concentration gradient. Occupancy of the phosphorylation site by vanadate, which acts presumably as an analogue of a pentacoordinated enzyme-phosphate transition complex with the enzyme, also reduces the affinity of the ATPase for calcium [22–24].

During the last few years, the intrinsic tryptophan fluorescence of the sarcoplasmic reticulum ATPase has been extensively used to monitor calcium occupancy of the sites in the high-affinity state [11,13,14,31–33]. With our present experiments we have found that the rise of intrinsic fluorescence following addition of calcium to sarcoplasmic reticulum ATPase preincubated with vanadate in the absence of calcium, occurs at very slow rates which are comparable to those of vanadate dissociation from the enzyme [23,34]. Such a slow reequilibration permits kinetic characterization of the calcium sites in the presence of non-saturating vanadate, demonstrating that these residual sites display exactly the same behavior as the high-affinity sites in the absence of vanadate (Figs. 3 and 4). These findings are consistent with a competitive inhibition of vanadate on high-affinity calcium binding, as already indicated by equilibrium experiments with radioactive tracer [24,25].

It is of great interest that the intrinsic fluorescence of ATPase preincubated with vanadate can be raised with mM (rather than with μM) Ca^{2+}, and the rates of calcium-dependent fluorescence rise in the presence of vanadate are faster when calcium is added to 'leaky' than to 'native' vesicles. This behavior can be attributed to a reduction in affinity and a change in orientation of the calcium binding sites as a consequence of vanadate binding to the ATPase.

The reaction of the sarcoplasmic reticulum ATPase with vanadate and calcium can be represented with the following simple scheme:



Scheme I.

where E and *E represent the ATPase with calcium sites in the high-affinity and outward-oriented state, or low-affinity and inward-oriented state, respectively; Va stands for vanadate. $*EVaCa_2$ is assumed to be unstable, and ECa_2 to yield high-intrinsic fluorescence. Following preincubation with vanadate in the absence of calcium, the enzyme resides either in E or *EVa. In the simplest case, addition of calcium in the micromolar range produces ECa_2 through reaction 1, and the velocity of ECa_2 accumulation (revealed by the fluorescence rise) is mostly dependent on the slow rate of vanadate dissociation through reaction 4, and the concentration of E which in the presence of saturating vanadate is very low.

It is apparent on Scheme I that in addition to binding to the high-affinity sites, vanadate dissociation can be initiated by calcium binding to sites in the low-affinity and inward-oriented state (*EVa, induced by vanadate complexation with the enzyme) and reequilibration of the various species. In fact, when high calcium concentrations are added to enzyme saturated with vanadate in the absence of calcium, the maximal fluorescence rise (which is normally observed in the absence of vanadate) is obtained relatively fast when the sarcoplasmic reticulum membrane is rendered permeable to Ca^{2+}. The mM K_d for Ca^{2+} indicates that these are the sites that have been transformed into a low-affinity state as a consequence of vanadate binding to the enzyme, and can be reconverted into the high-affinity (and high fluorescent-) state after vanadate dissociation from the phosphorylation site. When Ca^{2+} is added at concentrations (mM) that saturate only partially the low-affinity sites, the fluorescence rise is diphasic (Fig. 7), with a fast component due to vanadate dissociation directly through reaction 2, and a slow component due to calcium binding to the low concentration of E and vanadate displacement through reaction 4.

In conclusion, our measurements of intrinsic fluorescence are in general agreement with the measurements of radioactive calcium reported by Medda and Hasselbach [23,34], and demonstrate that vanadate binding to sarcoplasmic reticulum ATPase induces a reversible conversion of the specific calcium binding sites from a high-affinity and outward-oriented state to a low-affinity and inward-oriented state. The intrinsic fluorescence measurements are of considerable advantage in experiments on the kinetics of interconversion.

Finally, it should be pointed out that all our experimental observations are to be attributed to the effect of monovanadate, since the pH (8.0) and the low-concentration of our vanadate stock solution prevents formation of polyvanadate species.

Acknowledgments

We would like to thank Drs. Gómez Fernández and García Carmona for helpful discussions. This research was supported by grants from the National Institutes of Health (HL-27867) and the Muscular Dystrophy Association. F.F.B. was a Postdoctoral Fellow of the Muscular Dystrophy Association of America.

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