VANADATE INHIBITION OF THE Ca^{2+}-DEPENDENT CONFORMATIONAL CHANGE OF THE SARCOPLASMIC RETICULUM Ca^{2+}-ATPase

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1. Introduction

Vanadate has been shown to be a potent inhibitor of many phosphohydrolases which include a covalent phosphoenzyme intermediate in their enzymatic cycle [1]. The similar structure of pentavalent vanadium and phosphorus was suggested to be responsible for this inhibitory effect [2]. Extensive work has been done on the inhibitory effect of vanadate on the (Na^+,K^+)-ATPase [3–5]. Binding to the phosphorylation site is facilitated by K^+ and Mg^{2+} and locks the enzyme in the E_2 state [5–6]. Vanadate inhibition of Ca^{2+}-ATPase activity of sarcoplasmic reticulum has been reported in [7,8], the apparent affinity for the inhibitor being however much lower than for the (Na^+,K^+)-ATPase.

These results prompted us to investigate the effect of vanadate on the major conformational change of the Ca^{2+}-ATPase which is induced by Ca^{2+} binding to the high affinity sites. This change can be followed by intrinsic fluorescence measurements [9]. The main conclusions of this report are:

(i) Vanadate binds with high affinity ($K_d \simeq 0.2\ \mu M$) to the calcium free state E of the Ca^{2+}-ATPase;
(ii) The binding is a slow process which requires Mg^{2+} and is competitively antagonized by orthophosphate;
(iii) Binding of vanadate results in stabilizing the calcium free conformation of the Ca^{2+}-ATPase.

Abbreviations: Mops, morpholinopropane sulfonic acid; EGTA, ethylene glycol-bis (amino-2-ethylether), *N,N,N',N'*-tetraacetic acid

2. Materials and methods

2.1. *Preparation of sarcoplasmic reticulum membranes*

Sarcoplasmic reticulum vesicles were prepared from rabbit muscle as in [10] with an additional incubation in 0.6 M KCl before the final washing steps. Protein concentration was determined either as in [11] by comparison with bovine serum albumin or spectrophotometrically at 280 nm in the presence of 1% SDS as in [12]. Both methods give comparable results.

2.2. *Fluorescence measurements*

Fluorescence changes were measured in a 1 × 1 cm fluorescence cuvette with continuous stirring. Reagents were injected with Hamilton syringes in very small volumes (2–10 μl). Tryptophan was excited at 295 nm with a 75 W Xe (Hg) arc lamp through a Zeiss M4 Q III monochromator. The emitted light was measured at right angle through a Corning 0.54 cut-off filter.

2.3. *Chemicals*

Sodium orthovanadate ($Na_3VO_4 \cdot 14\ H_2O$, containing 22–25% insoluble V_2O_5 according to the manufacturer) was obtained from British Drug Houses. Concentration of vanadium in the solutions used was measured by atomic absorption (Laboratoire d'Analyses du Centre d'Etudes Nucléaires de Grenoble) and found significantly higher than expected from gravimetric measurements. Because of these uncertainties we estimate in this work the [vanadate] to be within ±30% of the indicated values.

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3. Results

3.1. *Effect of vanadate on the Ca^{2+}-dependent fluorescence change*

The conformation changes induced by calcium binding to and release from the high affinity cytoplasmic sites are followed by intrinsic fluorescence measurements as in [9]. The EGTA-induced fluorescence drop corresponding to the $E^{\dagger} \cdot Ca \rightarrow E + Ca^{2+}$ transition is unaffected by prolonged incubation in vanadate. The reverse transition $E \rightarrow E^{\dagger}$ normally induced by addition of μM levels of free calcium is however extremely slow in the presence of vanadate and Mg^{2+} (fig.1) indicating a stabilization of the calcium free E state.

At low [vanadate] part of the fast Ca^{2+}-induced change is observed indicating that only partial inhibition of the $E \rightarrow E^{\dagger}$ transition has occurred. Measurements of the extent of inhibition after prolonged incubation in EGTA and various concentrations of vanadate indicate a very high apparent affinity for the vanadate effect (fig.2). The number of active binding sites and the affinity for vanadate can be estimated using a representation analogous to that in [13]. The linear plot obtained is consistent with the existence of one single binding site of app. $K_d \simeq 250$ nM. The

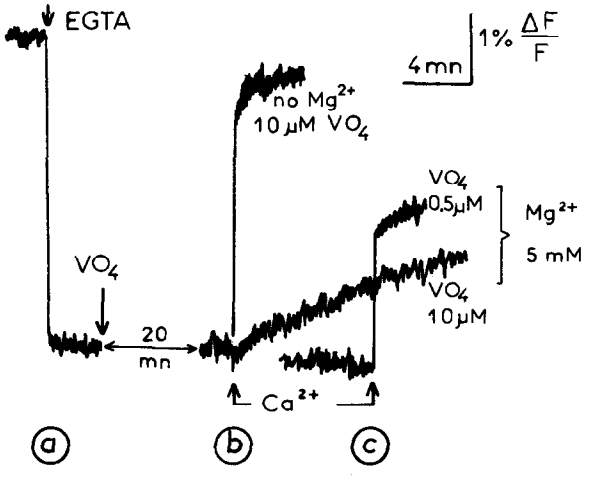

Fig.1. Intrinsic fluorescence changes of the Ca^{2+}-ATPase induced by: (a) removal of Ca^{2+} by 250 μM EGTA; (b,c) restoring a high [Ca^{2+}] by injection of 500 μM Ca^{2+}. In the presence of 5 mM Mg^{2+} the fast fluorescence increase is totally inhibited by 10 μM vanadate. Partial inhibition by 0.5 μM VO_4 is observed in (c). Protein is 100 μg/ml in 100 mM KCl, 20 mM Mops, pH 7.2, 20°C. Integration time constant is τ = 50 ms.

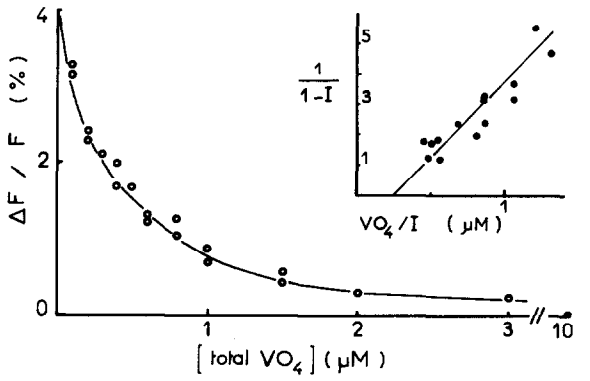

Fig.2. Measurement of the fast Ca^{2+}-induced fluoresence increase in the presence of 5 mM Mg^{2+} at varying [vanadate]. The same data have been used for the plot shown in the inset. If inhibition is produced by vanadate binding to one single site the relation between the fraction of enzyme inhibited (I) and the total concentration of vanadate is given by:

$$\frac{1}{1-I} = \frac{1}{K_i}\frac{[VO_{4,\,total}]}{I} - \frac{[n_i]}{K_i} \qquad [13]$$

Consistently with the existence of one single site a straight line can be drawn through the experimental points. Its slope gives an estimate of the dissociation constant of the vanadate–enzyme complex ($K_i \simeq 250$ nM); intersection with the abscissa axis gives the concentration of inhibitory sites ($[n_i] \simeq 0.25$ μM). Conditions are identical to those in fig.1. Membranes are incubated for 90 min in EGTA and vanadate before measuring the Ca^{2+}-induced fluorescence change.

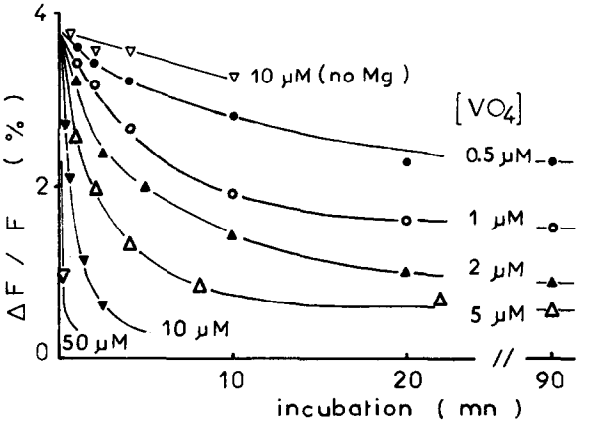

Fig.3. Time course of inhibition of the Ca^{2+}-induced fluorescence increase. The amplitude of the fast fluoresence change is plotted as a function of the duration of incubation in 250 μM EGTA and 5 mM Mg^{2+} at various vanadate concentrations (total concentration is indicated in the figure). A control experiment (▽) indicates that the inhibition requires the presence of Mg^{2+}. Preincubation in Ca^{2+} has no effect on the time course of inhibition. Conditions are identical to those in fig.1.

number of sites obtained is: 2.5 ± 0.7 nmol/mg protein, the precision being limited mostly by the uncertainties on [orthovanadate] (see section 2). This value is of the same order as the number of phosphorylation and ATP binding sites found in the same enzyme preparation: 3 nmol/mg [14].

3.2. *Time course of vanadate binding*

Inhibition of the Ca^{2+}-induced conformational change by vanadate is a very slow process. Fig.3 shows the extent of inhibition of the Ca^{2+}-induced fluorescence change as a function of the duration of exposure to vanadate in the absence of calcium. Preincubation in the presence of calcium has no effect on the kinetics of inhibition whatever the [vanadate]. The time course of inhibition is always very close to a single exponential process and the rate constant obtained is proportional to the vanadate up to 100 μM where it becomes too fast to be measured. This suggests a bimolecular association between vanadate and 1 single site on the calcium free enzyme.

3.3. *Competition with orthophosphate*

Titration of the inhibitory effect of vanadate on the Ca^{2+}-induced fluorescence change was repeated in in the presence of various concentrations of phosphate. Phosphate is able to prevent the vanadate effect. The double inverse plot in fig.4 is characteristic of a competitive binding indicating that vanadate is bound at the phosphorylation site of the Ca^{2+}-ATPase. A K_d for phosphate of ~25 mM can be deduced from this experiment in good agreement with values obtained from phosphorylation studies [15,16].

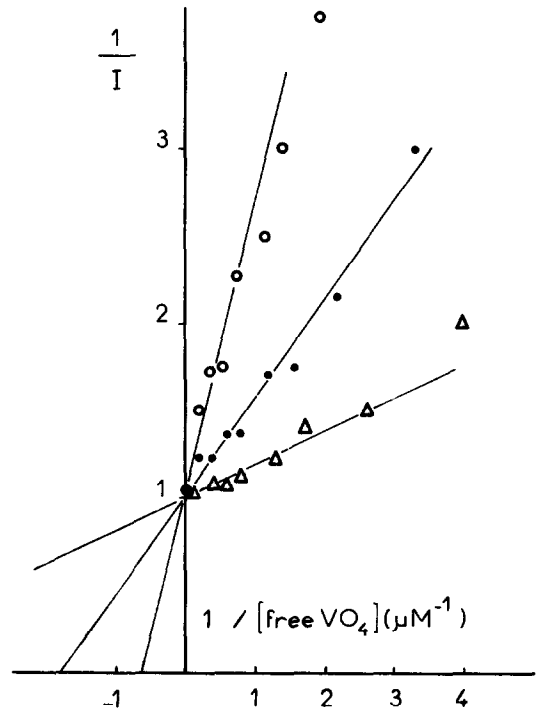

Fig.4. Competition between vanadate and phosphate. The inverse of the extent of inhibition (I) of the Ca^{2+}-induced fluorescence change is plotted as a function of the inverse of the free vanadate concentration at various total phosphate concentrations: (△) no PO_4; (•) 50 mM and (○) 100 mM PO_4. The free vanadate concentration is calculated by subtracting the fraction bound: $I \cdot [n_i]$ from the total vanadate concentration (the concentration of inhibitory sites $[n_i]$ being taken as 0.25 μM, see fig.2).

4. Discussion

These results presented show that, in the presence of Mg^{2+}, vanadate binds with high affinity to the calcium free E state of the Ca^{2+}-ATPase. The Ca^{2+}-induced conformational change $E \rightarrow E^{\dagger}$ is considerably slowed down suggesting that binding of vanadate results in a stabilization of the E state. This effect of vanadate resembles the stabilizing effect of phosphate on the same calcium free state under conditions where a stable phosphate bond is formed [17,18]. The observation of a competition between vanadate and phosphate, and the titration of a similar number of sites for vanadate binding and phosphorylation strongly suggests that stabilization of the calcium free conformation is due to the formation of a stable vanadate–enzyme bond E–Mg–V at the site of phosphorylation.

An analogous mechanism was proposed for the inhibition of the (Na^+,K^+)-ATPase [5]. Using a fluoresceine-labelled enzyme [6] have confirmed vanadate binding to the (Na^+,K^+)-ATPase was confirmed to stabilize the E_2K conformation of the enzyme.

A 50% inhibition of the steady state rate of the Ca^{2+}-ATPase was obtained at ~10 μM vanadate or above which is much higher than the affinity for the E state measured here [7,8]. This weak inhibitory effect on the ATPase activity, confirmed by us (not shown), indicates that most of the steady state intermediates formed are less susceptible to vanadate than the calcium free E conformation, being either the E–Mg–P or the $E^{\dagger} \cdot Ca$ species. In addition competi-

tion with ATP is likely to occur [5] for the (Na^+,K^+)-ATPase.

Our results do not support the hypothesis [17] that vanadate binding is prevented by internal calcium since inhibition of the Ca^{2+}-induced conformational change of the enzyme was observed in undepleted vesicles.

Acknowledgement

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