A kinetic study of the interaction of vanadate with the $\text{Ca}^{2+} + \text{Mg}^{2+}$ -dependent ATPase from sarcoplasmic reticulum

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$\text{Ca}^{2+} + \text{Mg}^{2+}$ -dependent ATPase from sarcoplasmic reticulum was inhibited by preincubation with vanadate. When the inhibited enzyme was preincubated in the presence of vanadate and assayed in its absence, a slow reactivation process was observed. This slow, hysteretic, process was exploited to study the influence of Ca^{2+} and ATP on the dissociation of vanadate. Ca^{2+} alone slowly displaced vanadate from the inhibited enzyme, and a rate constant of 0.1 min^{-1} , at 25°C , was calculated for this re-activation process. However, ATP re-activated with an apparent constant that hyperbolically depended on ATP concentration, and from it a rate constant for vanadate dissociation induced by ATP of 0.5 min^{-1} was calculated. It is deduced from the kinetic studies that ATP binds to the enzyme–vanadate complex, forming a ternary complex, with a dissociation constant of $4 \mu\text{M}$, and that this binding accelerates vanadate dissociation. Binding experiments with $[^{14}\text{C}]\text{ATP}$ showed that ATP binds to the enzyme–vanadate complex with a dissociation constant of $12 \mu\text{M}$, i.e. the affinities calculated with the isotope technique and the kinetic procedure are of the same order of magnitude.

The hydrolytic cycle of the Ca^{2+} -ATPase from sarcoplasmic reticulum has been described (Guilain *et al.*, 1981) by the minimal scheme shown in Scheme 1, where *E and E are the P_i -reactive and ATP-reactive conformations respectively of the unphosphorylated forms.

Several authors have previously shown that vanadate can inhibit the sarcoplasmic-reticulum $\text{Ca}^{2+} + \text{Mg}^{2+}$ -dependent ATPase (O'Neal *et al.*, 1979; Wang *et al.*, 1979; Pick, 1982; Pick & Karlsh, 1982; Dupont & Bennet, 1982). Vanadate inhibits, in general, different phosphohydrolases having in common a covalent phosphoenzyme intermediate in their catalytic cycles (Macara, 1980). Although the affinity of this inhibitor for the $\text{Ca}^{2+} + \text{Mg}^{2+}$ -dependent ATPase is lower than for other ATPases, for example the $\text{Na}^+ + \text{K}^+$ -dependent ATPase (O'Neal *et al.*, 1979), it is still a useful

tool for the investigation of some of the steps of the enzymic cycle of the sarcoplasmic-reticulum Ca^{2+} pump.

The mechanism of action of vanadate has been proposed to be the formation of a transition state analogous to that of phosphate hydrolysis because of its ability to exist in a stable trigonal bipyramidal structure (Lindquist *et al.*, 1973; Van Etten *et al.*, 1974; López *et al.*, 1976). Therefore we should expect binding of vanadate to the *E form of the ATPase, which is precisely the form phosphorylated by P_i . This is indeed what has been found,

Scheme 1

Abbreviations used: ATPase or Ca^{2+} -ATPase, the $\text{Ca}^{2+} + \text{Mg}^{2+}$ -dependent ATP-hydrolysing enzyme from sarcoplasmic-reticulum membranes; Tes, 2-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]ethanesulphonic acid.

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since vanadate inhibition is only observed in the absence of Ca^{2+} and in the presence of Mg^{2+} (O'Neal *et al.*, 1979; Wang *et al.*, 1979; Pick, 1982; Pick & Karlsh, 1982; Dupont & Bennet, 1982). Furthermore, competition of binding between vanadate and P_i has been reported by observing the inhibition by vanadate of the Ca^{2+} -dependent conformational change of the enzyme (Dupont & Bennet, 1982) and by measuring the phosphorylation of the ATPase in the absence of Ca^{2+} (Pick, 1982).

In the present paper we report our studies on the re-activation of the Ca^{2+} -ATPase from sarcoplasmic reticulum inhibited by preincubation with vanadate. This re-activation is compared with the reaction of dephosphorylation of the ATPase, assuming that the enzyme–vanadate complex is analogous to the phosphorylated enzyme. We exploit the slowness of the re-activation process to study the influence of Ca^{2+} and ATP on its kinetics, and we propose a mechanism for this reaction.

Materials and methods

Chemicals

Sodium orthovanadate, vanadate-free ATP, NADH, phosphoenolpyruvate, pyruvate kinase and lactate dehydrogenase were from Sigma Chemical Co., Poole, Dorset, U.K. [^{14}C]ATP was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. All other chemicals were of analytical grade.

Analytical assays

Protein was determined either by the method of Lowry *et al.* (1951) with bovine serum albumin as standard, or from the ATPase molar absorption (Hardwicke & Green, 1974).

ATPase activity was assayed at 25°C by using an ATP-regenerating system essentially as described previously (Gómez-Fernández *et al.*, 1980), containing 100 mM-KCl, 5 mM- MgCl_2 , 0.6 mM-EGTA, 0.2 mM-NADH, 2 mM-phosphoenolpyruvate, 4 units ($\mu\text{mol}/\text{min}$) of pyruvate kinase/ml, 6 units ($\mu\text{mol}/\text{min}$) of lactate dehydrogenase/ml, 2 mM-MgATP (unless otherwise stated) and CaCl_2 to give 4 μM free Ca^{2+} .

Free Ca^{2+} concentration was calculated from total Ca^{2+} and EGTA by computations (Fabiato & Fabiato, 1978, 1979) taking into account pH, Mg^{2+} concentration, phosphoenolpyruvate, P_i and nucleotides when present. The Ca^{2+} present in the solutions as contaminant was not considered in calculations, since it was assessed to be lower than 1 μM by atomic absorption spectrophotometry.

$\text{Ca}^{2+} + \text{Mg}^{2+}$ -dependent ATPase

Sarcoplasmic reticulum was prepared from rabbit back and leg white skeletal muscles by the method of Nakamura *et al.* (1976) but with inclusion of 1 mM-dithiothreitol and 5 μM -phenylmethanesulphonyl fluoride in all the buffers. It was finally resuspended in 1 M-KCl/0.25 M-sucrose/50 mM-Tes/NaOH buffer, pH 8.0, and rapidly frozen and stored, in small portions, in liquid N_2 . It was used within 2 weeks of its preparation.

Uncoupled ATPase

EGTA-uncoupled vesicles were prepared as described by Diamond *et al.* (1980); sarcoplasmic-reticulum vesicles, at a protein concentration of 1.5 mg/ml, were incubated at 37°C for 75 min in 100 mM-KCl/5 mM-EGTA/50 mM-Tes buffer, pH 7.0. It was observed that the initial specific activity of the vesicles of 0.8–1.0 $\mu\text{mol}/\text{min}$ per mg, at 25°C and 2 mM-MgATP, increased after incubation with EGTA to about 2.0 $\mu\text{mol}/\text{min}$ per mg. If the uncoupled sample was then kept chilled at 0–4°C, the activity remained constant for at least 4 h, which was the maximum period of time needed for the experiments.

Preincubation with vanadate

Uncoupled sarcoplasmic-reticulum vesicles, at a concentration of 1.5 mg/ml, were preincubated with various concentrations of vanadate, usually 200 μM , in a 100 mM-KCl/5 mM- MgCl_2 /5 mM-EGTA/50 mM-Tes buffer, pH 7.0. Samples from this preincubation mixture were used to assay the activity after a given preincubation period. The 100 μl samples were freed from excess vanadate by centrifugation at 4°C, through Sephadex G-50 (fine grade), packed in 1 ml tuberculin syringes, in accordance with the general method of Penefsky (1977) to desalt proteins and as described by Pick (1982) to remove excess vanadate from sarcoplasmic-reticulum vesicles. Excess vanadate should be completely removed since the Ca^{2+} -ATPase treated in this way might be fully re-activated. The gel was previously equilibrated with the same buffer used for the preincubation. Samples were assayed for ATPase activity within 5 min after centrifugation. Within this period, no significant decrease in vanadate inhibition was observed.

ATP binding

[^{14}C]ATP binding to the enzyme–vanadate complex was assayed in the absence of calcium at 25°C essentially as described by Dupont (1977) for binding of ATP to Ca^{2+} -ATPase. Sarcoplasmic-reticulum vesicles (0.4 mg of protein) uncoupled by the EGTA treatment, as described above, were

preincubated in 2 ml of a $200\text{ }\mu\text{M}$ -vanadate in $100\text{ mM-KCl}/5\text{ mM-EGTA}/50\text{ mM-Tes}$ buffer, pH 7.0.

After 10 min of preincubation [^{14}C]ATP (555 c.p.m./nmol of ATP) was added to the desired concentration and the suspension immediately filtered through a $0.45\text{ }\mu\text{m}$ -pore size Sartorius filter. Washing of the filters not being possible, the blank was obtained by filtering the same solution without proteins. As previously described by Dupont (1977), it was observed that for ATP concentrations higher than approx. $400\text{ }\mu\text{M}$ the blanks represent as much as 80% of the total radioactivity measured, and hence the assay becomes unreliable. All assays were made in duplicate, and mean values were used for calculations.

Results

Preincubation of uncoupled sarcoplasmic-reticulum vesicles with vanadate, in the absence of Ca^{2+} and in the presence of Mg^{2+} , resulted in non-linear plots of ATPase activity when the enzyme was assayed in the absence of vanadate. Fig. 1 shows that an initial lag period in the activity was observed. However, after this period the activity reached a steady-state rate identical with that of the control that had not been preincubated with vanadate. Increasing concentrations of vanadate gave a concomitant increase in the lag periods (Fig. 1). In agreement with previous reports (Pick, 1982; Dupont & Bennet, 1982), the presence of Mg^{2+} in the preincubation medium was essential for ATPase inhibition (results not shown).

Fig. 1. Re-activation of Ca^{2+} -ATPase inhibited by vanadate

Sarcoplasmic-reticulum vesicles, uncoupled by treatment with EGTA, were preincubated at a concentration of 1.5 mg/ml with different concentrations of vanadate. After 5 min, excess of vanadate was eliminated and the enzyme activity was assayed, $15\text{ }\mu\text{g}$ of protein being used. The spectrophotometric traces obtained, for the concentrations of vanadate indicated, are shown.

Pick (1982) suggested that both Ca^{2+} and ATP inhibited the binding of vanadate to the ATPase, and therefore that an interpretation of the observed time course of ATPase activity is that the substrates of the enzyme slowly displace vanadate from an enzyme-vanadate complex formed during the preincubation.

The time required for a maximum lag period was studied, and it was found that the maximum lag period was already observed after about 1 min of preincubation with vanadate (results not shown). In all the following experiments, a preincubation of 5 min was always carried out.

In most of the following experiments, $200\text{ }\mu\text{M}$ -vanadate was used. Fig. 2 shows that the maximum lag period was already obtained at this concentration, and a saturation process is observed.

The slow process of re-activation of ATPase inhibited by vanadate during its assay is a hysteretic process as defined by Frieden (1970). This property was exploited for the kinetic study of the re-activation of the ATPase induced by Ca^{2+} and ATP, as described in the following paragraphs.

Fig. 2. Lag period of the activity as a function of the concentration of vanadate: calculation of the dissociation constant of vanadate

Sarcoplasmic-reticulum vesicles, uncoupled by treatment with EGTA, were preincubated at a concentration of 1.5 mg/ml with different concentrations of vanadate. After 5 min, excess of vanadate was removed, and $15\text{ }\mu\text{g}$ of protein was added to the assay medium contained in a cuvette. Lag periods of the activity were measured from the spectrophotometric traces, as indicated in Fig. 1 legend. A double-reciprocal plot of the same data is shown in the insert. From it K_v (dissociation of vanadate) was calculated.

Calculation of the vanadate inhibition constant (K_V)

Assuming that the process of binding of vanadate is:

and defining K_V as the dissociation constant of vanadate, $[E_T]$ the total concentration of enzyme, $[E]$ the concentration of free enzyme and $[E \cdot V]$ the concentration of the enzyme form bearing bound vanadate, it follows that:

$$[E \cdot V] = \frac{[E_T] \cdot [V]}{K_V + [V]} \quad (i)$$

By using eqn. (9) of the Appendix, the following expression is obtained:

$$L = \frac{L_m \cdot [V]}{K_V + [V]} \quad (ii)$$

where L is the lag period and L_m is the maximum period of lag observed.

K_V and L_m can be calculated from a plot of $1/L$ versus $1/[V]$ (Fig. 2, insert).

For the ATPase uncoupled by preincubation with EGTA K_V was found to be $28 \mu\text{M}$, and when 2mM -ATP was used in the enzyme assay L_m was 1.6min .

Re-activation of ATPase, preincubated with vanadate, by Ca^{2+}

ATPase preincubated with $200 \mu\text{M}$ -vanadate in the absence of Ca^{2+} but in the presence of Mg^{2+} was incubated in the regenerating assay medium from which ATP was initially excluded although it contained $4 \mu\text{M}$ free Ca^{2+} . After different incubation periods the enzymic reaction was initiated by the addition of 2mM -MgATP. Fig. 3 shows that the extent of the lag period observed in the activity before reaching the steady state depends on the time of incubation with Ca^{2+} . Longer periods of incubation yielded shorter lag periods in the activity. The process of re-activation, i.e. decrease in the lag period, was rather slow; 10min of incubation were necessary to decrease the lag period from 45s (without incubation with Ca^{2+} at all) to 15s .

The re-activation during the assay with 2mM -ATP is faster than the re-activation by Ca^{2+} during the incubation without ATP. As shown below, the reason is that ATP speeds up the process of re-activation.

Calculation of the apparent rate constant of re-activation of the ATPase in the presence of Ca^{2+} and ATP

Before calculation of the time-constant of re-activation by Ca^{2+} it is important to study the re-activation produced during the enzyme assay, i.e.

Fig. 3. Dependence of lag periods of the activity on time of incubation with Ca^{2+}

Sarcoplasmic-reticulum vesicles, uncoupled by treatment with EGTA, were preincubated at a concentration of 1.5mg/ml with $200 \mu\text{M}$ -vanadate. After 5min , excess of vanadate was removed, and $15 \mu\text{g}$ of protein was added to the assay medium containing $4 \mu\text{M}$ free Ca^{2+} and from which ATP was omitted. The incubation in this medium proceeded for different times, and was terminated by addition of 2mM -ATP. The activity was then recorded. The plot of the logarithms of the lag periods versus incubation times is shown in the insert.

in the presence of ATP and Ca^{2+} . It should be checked whether it follows first-order kinetics and what is its dependence on the time of incubation with Ca^{2+} in the absence of ATP.

The apparent time-constant of re-activation in the presence of ATP ($k_{app.}$) was studied by using the activity traces obtained after incubations with Ca^{2+} for different periods.

It is shown in the Appendix (eqn. 5) that for a hysteretic enzyme reaction it is true that:

$$\ln(P_t - P_s) = \ln\left(\frac{v_t - v_0}{k_{app.}}\right) - k_{app.} t$$

where P_s is the product concentration for the steady-state asymptotic line, P_t is observed product concentration at time t , v_t is the steady-state rate and v_0 the initial rate. A plot of $\ln(P_t - P_s)$ versus time yields a straight line if the reaction follows first-order kinetics, and $k_{app.}$ may be deduced from its slope. Fig. 4 shows that straight lines were indeed obtained when the assays were made after different times of incubation with Ca^{2+} . The lines were parallel, indicating that $k_{app.}$ was the same in all the cases. The value obtained for $k_{app.}$ was $0.5 \pm 0.02\text{min}^{-1}$. $k_{app.}$ depends on the concentration of ATP used in each assay, as is shown below.

Fig. 4. Kinetic analysis of the lag period of the activity $P_t - P_s$ values were calculated from data of Fig. 3. P_t is the amount of product actually accumulated at each particular time t , and P_s is the value of the asymptotic straight line of the curve of accumulation of product at each particular time. Measurements corresponding to different times of preincubation with Ca^{2+} are shown: $\circ$, without preincubation with Ca^{2+} ; $\bullet$, 0.5 min; $\triangle$, 1 min; $\blacktriangle$, 5 min; ∇ , 10 min of preincubation with Ca^{2+} .

Obviously, the re-activation induced by 2 mM-ATP followed a molecular mechanism that was independent of the time of preincubation with Ca^{2+} alone. A feasible interpretation is that Ca^{2+} does not re-activate the enzyme by slowly creating new chemical species such as ternary vanadate-enzyme- Ca^{2+} complexes. If these complexes were formed, k_{app} would change with time of incubation, i.e. with increasing concentrations of the new species. This is also confirmed by the experiment of Table 1, where k_{app} was calculated for several concentrations of free Ca^{2+} in the assay medium. It is shown in Table 1 that k_{app} remained constant when pCa of free Ca^{2+} was varied between 7.69 and 4.15.

However, ATP has a protective effect during the incubation of the ATPase with vanadate. The lag period was decreased when increasing concentrations of ATP (between 0 and 500 μM) were included during the incubation of the ATPase with concentrations of vanadate ranging between 30 and 200 μM (results not shown).

Table 1. Dependence of k_{app} of the re-activation on the concentration of free Ca^{2+}

The enzyme was inhibited by preincubation with 200 μM -vanadate. Conditions of preincubation and of ATPase activity measurements were as indicated in Fig. 5 legend, except that different concentration of free Ca^{2+} were used whereas ATP concentration was kept at 2 mM in all the cases.

pCa	k_{app} (min^{-1})
7.69	0.47 ± 0.02
6.88	0.45
6.25	0.45
4.57	0.47
4.15	0.45

Calculations of the rate constant of displacement of vanadate by Ca^{2+}

Since Ca^{2+} does not bind to the enzyme-vanadate complex, it should operate by binding to the free enzyme. According to Scheme 1, above, Ca^{2+} binding shifts the equilibrium from the $*E$ to the E conformation of the enzyme, as shown in Scheme 2, where V represents orthovanadate. Ca^{2+} shifts the equilibrium towards the right and hence it will re-activate the enzyme. This interpretation agrees with previous suggestions for the re-activation of vanadate-inhibited ATPase by Ca^{2+} (Pick, 1982; Dupont & Bennet, 1982).

In accordance with Scheme 2 it is deduced in the Appendix (eqn. 11) that:

$$\ln L = \ln L_m - k_v \cdot t$$

where L is the lag period observed during the assay of the activity, L_m is the maximum lag period and t is the time of incubation with Ca^{2+} before addition of ATP to start the assay. After plotting $\ln L$ versus time of incubation with Ca^{2+} , k_v was calculated from the slope of the straight line (Fig. 3, insert). Its value was $0.1 \pm 0.005 \text{ min}^{-1}$. This is a relatively slow process, with a $t_{\frac{1}{2}}$ of 6.9 min.

Influence of ATP concentration on the re-activation process

Uncoupled sarcoplasmic-reticulum vesicles were preincubated in the presence of 200 μM -vanadate and in the absence of Ca^{2+} for 5 min. After elimination of excess vanadate, portions were taken for assay of ATPase activity with different concentrations of ATP. By using eqn. (4) of the Appendix, k_{app} of the re-activation process

were calculated from the reciprocals of the lag period of the activity for each ATP concentration. This is valid, since 200 μM -vanadate is a saturating concentration and initial rates were indeed observed to be zero.

Fig. 5 shows that k_{app} hyperbolically depends on ATP concentration. These results suggest that ATP induces a displacement of vanadate from the enzyme–vanadate complex, hence re-activating the enzyme.

The simplest mechanism that can account for these observations is the formation of a ternary complex between enzyme, vanadate and ATP, as shown in Scheme 3.

Scheme 3

Fig. 5. Apparent constant of re-activation induced by ATP as a function of the concentration of ATP

Sarcoplasmic-reticulum vesicles, uncoupled by treatment with EGTA, were preincubated at a concentration of 1.5 mg/ml with 200 μM -vanadate. After 5 min, excess of vanadate was removed, and 15 μg of protein was added to the spectrophotometric cuvette containing the assay medium. Different ATP concentrations were present in this assay medium. k_{app} of re-activation was calculated for each ATP concentration as the reciprocals of the lag periods. The insert shows the calculation of the dissociation constant of ATP from the enzyme–vanadate complex. The values of $1/(k_{\text{app}} - k_v)$ for each ATP concentration were calculated. k_v corresponds to re-activation by Ca^{2+} , in the absence of ATP, i.e. 0.1 min^{-1} as deduced from Fig. 3 (insert).

Assuming that the slow step in Scheme 3 is the dissociation of vanadate, this reaction was analysed by an equilibrium approach, and eqn. (20) of the Appendix was obtained:

$$k_{\text{app}} = k_v + (k_{+2} - k_v) \frac{[\text{ATP}]}{[\text{ATP}] + K_s}$$

where K_s is the ratio k_{-1}/k_{+1} . Hence k_{app} should depend on $[\text{ATP}]$ in a hyperbolic mode, as was indeed observed (Fig. 5).

According to eqn. (21) of the Appendix:

$$\frac{1}{k_{\text{app}} - k_v} = \frac{K_s}{k_{+2} - k_v} \cdot \frac{1}{[\text{ATP}]} + \frac{1}{k_{+2} - k_v}$$

and therefore a plot of $1/(k_{\text{app}} - k_v)$ versus $1/[\text{ATP}]$ allows the calculation of k_2 and K_s . Fig. 5 (insert) shows such a plot, and from it k_{+1} was calculated to be $0.5 \pm 0.02 \text{ min}^{-1}$ and K_s was equal to 4 μM .

k_{+2} is higher than k_v , indicating that ATP re-activates faster than Ca^{2+} .

Binding of ATP to the enzyme–vanadate complex

To confirm the binding of ATP to the enzyme inhibited by vanadate, uncoupled sarcoplasmic-reticulum vesicles were preincubated with 200 μM -vanadate, and then different concentrations of [^{14}C]ATP were added. The mixtures were immediately filtered on Sartorius filters and radioactivities were measured. The filtration was completed in less than 15 s, to ensure that vanadate was not dissociated from the enzyme during the experiment. Fig. 6 shows a Scatchard plot of the results. From this plot a K_d of 12 μM was deduced. This value is slightly higher than the 3 μM value obtained by Dupont (1977) for the high-affinity site, with unphosphorylated enzyme and in the absence of Ca^{2+} . It is also slightly higher than the 4 μM value obtained in the present work from kinetic measurements for the enzyme–vanadate complex. Nevertheless all these values are of the same order of magnitude, and taking into account the different approaches used to calculate them it might be concluded that all of them correspond to the same binding site.

It is deduced also from Fig. 6 that each 1 mg of ATPase binds 4.4 nmol of ATP. This binding capacity is identical with that found by Dupont (1977) for the high-affinity site.

As mentioned in the Materials and methods section, the procedure used in the present work to measure the binding of ATP to the enzyme does not reliably allow the measurement of a possible low-affinity site, which could have a K_d in the millimolar range.

Fig. 6. *Binding of ATP to the enzyme-vanadate complex* Uncoupled sarcoplasmic-reticulum vesicles were incubated with $20\ \mu\text{M}$ -vanadate at a concentration of 0.4 mg/ml . After 5 min, different concentrations of [^{14}C]ATP were added. To ensure that vanadate was bound to the enzyme, the excess of vanadate was not eliminated before the addition of ATP. The samples were immediately filtered on $0.45\ \mu\text{m}$ -pore-size Sartorius filters. The radioactivity was counted and the results were plotted in accordance with the Scatchard mode.

Discussion

The results presented here show that the interaction of vanadate with the ATPase is slow and that the inhibited complex gives a hysteretic type of response when it is re-activated by the natural substrates, Ca^{2+} and ATP.

The stability of the enzyme-vanadate complex is due to the slowness of the dissociation reaction of vanadate from this complex. This is an advantage of the use of vanadate, since phosphate dissociates from the enzyme very rapidly (Guillain *et al.*, 1981), hence making its kinetic study difficult.

Our results indicate, in agreement with previous authors (Pick, 1982; Pick & Karlish, 1982; Dupont & Bennet, 1982), that Ca^{2+} does not bind to the enzyme-vanadate complex, and therefore the re-activation induced by incubation with Ca^{2+} follows a mechanism in which vanadate has to dissociate first, through a slow process (see Scheme 2).

The conformational transition $*\text{E} \rightarrow \text{E}$ that follows the binding of Ca^{2+} is relatively slow when compared with other reactions of the enzymic cycle; rate constants for this transition of 5 s^{-1} at 22°C (Dupont & Leigh, 1978) and 10 s^{-1} (Guillain *et al.*, 1980) have been described for experimental conditions not very different from those used in the present work. Since our calculated k_v has a value of 0.1 min^{-1} at 25°C , the $*\text{E} \rightarrow \text{E}$ transition is certainly more rapid than the dissociation of vanadate from the enzyme in the absence of ATP.

Our most essential finding is that ATP re-activates the inhibited enzyme by following a mechanism with a k_{app} that hyperbolically depends on ATP concentration. The simplest interpretation is that ATP binds to the enzyme-vanadate complex, i.e. before vanadate dissociation. This interpretation is also supported by the experiment where the binding of ATP was measured.

Pick (1982) also studied the incubation of vanadate with the Ca^{2+} -ATPase. However, he assumed a simple competition between nucleotides and vanadate, and he neglected possible effects of ligands such as ATP on the dissociation of the enzyme-vanadate complex. It should be pointed out that he used mainly equilibrium experiments, and that the kinetic approach and also the binding experiments used in the present work are much more appropriate for the study of the formation of complexes between enzyme, vanadate and ATP.

Pick (1982) obtained a dissociation constant for vanadate of $34\ \mu\text{M}$, which is of the same order as our $28\ \mu\text{M}$ value. We noticed that this constant might be affected by the previous history of the sarcoplasmic-reticulum vesicles (A. Ortiz, unpublished work). For example, it is different for ATPase purified with the use of detergents or for the native sarcoplasmic reticulum. This may be the reason why other authors (Dupont & Bennet, 1982) obtained a much lower value ($0.25\ \mu\text{M}$) for coupled sarcoplasmic reticulum.

An important question is to clarify how the inhibited enzyme binds ATP, i.e. to which site of the enzyme-vanadate complex the nucleotide is bound.

It has been deduced from measurements of binding of ATP to the Ca^{2+} -ATPase and from the complex effects of ATP on the ATPase kinetics (Yamamoto & Tonomura, 1967; Inesi *et al.*, 1967; de Meis & Fialho de Mello, 1973; Vianna, 1975; Dupont, 1977; Neet & Green, 1977; Taylor & Hattan, 1979; Moller *et al.*, 1980) that this enzyme might have two different types of ATP-binding sites, one of them being the catalytic centre and the other a regulatory site. The active centre would have a K_s of $3\ \mu\text{M}$ and the regulatory site a K_s of $500\ \mu\text{M}$ (Dupont, 1977).

The dissociation constant of ATP from the ternary complex was calculated in the present work to be $4\ \mu\text{M}$ from the kinetic measurements and $12\ \mu\text{M}$ from the binding experiments. These values are very close to the lower K_s value for ATP ($3\ \mu\text{M}$) determined by Dupont (1977) and to the $5\text{--}6\ \mu\text{M}$ value found by Andersen *et al.* (1982).

It is possible that binding of ATP to the high-affinity site might be precluded by vanadate being bound to the same site. However, it has been suggested that, when the active centre of the Ca^{2+} -ATPase is phosphorylated, the adenosine recognition site is clearly dislocated from the aspartic acid residue where phosphate is bound (Pick, 1981; Pick & Bassilian, 1981). If this is true, ATP will be able to bind to the active site even if vanadate is previously bound to the same site. The binding of ATP would induce tensions in the structure favouring the dissociation of vanadate.

McIntosh & Boyer (1983), who used the technique of isotopic exchange, suggested that possibly ATP binds to the catalytic site after ADP departure but before E-P hydrolysis. This is in agreement with our suggestion if phosphate and vanadate are considered to be analogues.

It is suggested, in conclusion, that, although in the normal turnover cycle the $*\text{E} \rightarrow \text{E}$ transition is the rate-limiting step, in the presence of vanadate the $*\text{E} \cdot \text{V} \rightarrow * \text{E} + \text{V}$ step becomes rate-limiting and that $*\text{E} \cdot \text{V}$ has a high affinity for ATP, which probably binds to the active centre, accelerating the dissociation of vanadate.

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APPENDIX

Calculation of the apparent constant of reactivation (k_{app})

It can be obtained from the spectrophotometric traces of activity.

Following Frieden (1970), for a first-order process, it is true that:

$$P_t = v_f t = \frac{1}{k_{app}} (v_f - v_0) (1 - e^{-k_{app} t}) \quad (1)$$

and, when the time tends to infinite:

$$P_s = v_f t = \frac{v_f - v_0}{k_{app}} \quad (2)$$

where P_t is the observed product concentration at time t , P_s is the product concentration for the steady-state asymptote line, v_f is the steady-state rate and v_0 is the initial rate.

The lag period in the activity (L) is defined as:

$$L = \frac{v_f - v_0}{v_f \cdot k_{\text{app}}} \quad (3)$$

and if $v_0 = 0$:

$$L = \frac{1}{k_{\text{app}}} \quad (4)$$

Subtracting eqn. (2) from eqn. (1), and taking the logarithm:

$$\ln(P_t - P_s) = \ln\left(\frac{v_f - v_0}{k_{\text{app}}}\right) - k_{\text{app}} \cdot t \quad (5)$$

Therefore a plot of $\ln(P_t - P_s)$ versus time will give a straight line, for a first-order process, and k_{app} can be calculated from its slope.

Calculation of the time-constant of dissociation of vanadate (k_v)

According to Scheme 2 of the main paper, the rate constant of dissociation of vanadate from the enzyme–vanadate complex may be calculated as follows. When the enzymic reaction is initiated by the addition of ATP, after preincubation with vanadate in the absence of Ca^{2+} and after the subsequent incubation with Ca^{2+} , the enzyme can be found in different forms, such that:

$$[\text{E} \cdot \text{V}] = [\text{E}_T] - ([\text{E} \cdot \text{Ca}] + [\text{E}]) \quad (6)$$

where $[\text{E}_T]$ is the total amount of enzyme, $[\text{E} \cdot \text{V}]$ is the amount of enzyme that still has bound vanadate, $[\text{E} \cdot \text{Ca}]$ is the amount of the enzyme with bound Ca^{2+} and $[\text{E}]$ is the amount of free enzyme. The enzyme activity that was still inhibited when ATP was added (v_i) must be proportional to $[\text{E} \cdot \text{V}]$. The initial rate (v_0), i.e. the rate at time zero, must be proportional to $([\text{E} \cdot \text{Ca}] + [\text{E}])$, assuming that the limiting rate constant is k_v . Finally, the maximum possible rate (v_f) must be proportional to $[\text{E}_T]$.

Hence:

$$v_i = v_f - v_0 \quad (7)$$

According to eqn. (3) above:

$$L = \frac{v_f - v_0}{v_f \cdot k_{\text{app}}} = \frac{v_i}{v_f \cdot k_{\text{app}}} \quad (8)$$

As v_f is constant for a given experiment:

$$L = k'[\text{E} \cdot \text{V}] \quad (9)$$

where k' represents a constant. Therefore the lag period is proportional to the concentration of the enzyme–vanadate complex remaining at each time, during the incubation with Ca^{2+} .

Since, after Scheme 2 of the main paper, the dis-

sociation of vanadate from the enzyme–vanadate complex follows first-order kinetics:

$$[\text{E} \cdot \text{V}]_t = [\text{E} \cdot \text{V}]_0 \cdot e^{-k_v t} \quad (10)$$

where $[\text{E} \cdot \text{V}]_0$ and $[\text{E} \cdot \text{V}]_t$ are the concentrations of complex at times zero and t respectively, t being the time of incubation with Ca^{2+} . Substituting $[\text{E} \cdot \text{V}]_t$ by L and $[\text{E} \cdot \text{V}]_0$ by L_m (maximum period of lag) and taking the logarithm:

$$\ln L = \ln L_m - k_v t \quad (11)$$

Dependence of k_{app} on ATP concentration

According Scheme 3 of the main paper:

$$K_s = \frac{k_{-1}}{k_{+1}} = \frac{[\text{E} \cdot \text{V}][\text{ATP}]}{[\text{ATP} \cdot \text{E} \cdot \text{V}]} \quad (12)$$

where K_s is the dissociation constant of ATP from the ternary complex formed by enzyme, ATP and vanadate.

The rate of enzymic re-activation will be:

$$-\frac{d[\text{E}_i]}{dt} = \frac{d[\text{E}_A]}{dt} = k_{+2}[\text{ATP} \cdot \text{E} \cdot \text{V}] + k_v[\text{E} \cdot \text{V}] \quad (13)$$

where $[\text{E}_i]$ is the concentration of inhibited forms of enzyme and $[\text{E}_A]$ is the concentration of active forms of enzyme. Hence:

$$[\text{E}_i] = [\text{ATP} \cdot \text{E} \cdot \text{V}] + [\text{E} \cdot \text{V}] \quad (14)$$

$$[\text{E}_A] = [\text{E}] + [\text{E} \cdot \text{Ca}] + [\text{E} \cdot \text{ATP}] \quad (15)$$

where E represents free enzyme and $\text{E} \cdot \text{Ca}$ the enzyme form bearing bound Ca^{2+} .

The proportion of inactive enzyme present as ternary complex enzyme–ATP–vanadate can be represented by $\bar{Y}_s$ (fractional saturation function):

$$\bar{Y}_s = \frac{[\text{ATP} \cdot \text{E} \cdot \text{V}]}{[\text{ATP} \cdot \text{E} \cdot \text{V}] + [\text{E} \cdot \text{V}]} \quad (16)$$

Substituting eqn. (12) in eqn. (16):

$$\bar{Y}_s = \frac{[\text{ATP}]}{K_s + [\text{ATP}]} \quad (17)$$

Combining eqns. (13), (14), (16) and (17):

$$-\frac{d[\text{E}_i]}{dt} = \left(k_v + (k_{+2} - k_v) \frac{[\text{ATP}]}{K_s + [\text{ATP}]} \right) [\text{E}_i] \quad (18)$$

and by integration of eqn. (18):

$$[\text{E}_i] = [\text{E}_i]^0 \cdot \exp - \left(k_v + (k_{+2} + k_v) \frac{[\text{ATP}]}{K_s + [\text{ATP}]} \right) t \quad (19)$$

Therefore the apparent constant for the first-order reaction of dissociation of vanadate in the presence of both Ca^{2+} and ATP is:

$$k_{\text{app.}} = k_v + (k_{+2} - k_v) \frac{[\text{ATP}]}{K_s + [\text{ATP}]} \quad (20)$$

Rearrangement of eqn. (20) then gives:

$$\frac{1}{k_{\text{app.}} - k_v} = \frac{K_s}{k_{+2} - k_v} \cdot \frac{1}{[\text{ATP}]} + \frac{1}{k_{+2} - k_v} \quad (21)$$

Reference

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