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The role of Mg^{2+} and Ca^{2+} in the simultaneous binding of vanadate and ATP at the phosphorylation site of sarcoplasmic reticulum Ca^{2+}-ATPase

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The sarcoplasmic reticulum Ca^{2+}-ATPase was reacted with vanadate in the presence of Mg^{2+} and EGTA, and the effect of Ca^{2+}, Mg^{2+} and ATP on the kinetics of vanadate release from the enzyme vanadate complex was studied after dilution with vanadate-free media. Ca^{2+} increased, whereas ATP decreased the rate of vanadate release. In absence of free Mg^{2+} in the release media ATP was bound to the vanadate-reacted Ca^{2+}-ATPase with high affinity (K_d 4–5 μM), and full saturation with ATP resulted in complete inhibition of vanadate release. In media containing free Mg^{2+}, where ATP predominantly was present as MgATP, binding of the nucleotide to vanadate-reacted Ca^{2+}-ATPase occurred with low apparent affinity. Mg^{2+} alone did not affect the rate of vanadate release. At saturating ATP concentrations the release rate in the presence of free Mg^{2+} was less inhibited than in its absence. These results indicate that uncomplexed ATP interacts with the same Mg^{2+} at the catalytic site, which is involved in formation of the enzyme-vanadate complex (EMgV), and thereby hinders dissociation of vanadate. Destabilization of the complex by free Mg^{2+} may be caused by the presence of an additional magnesium ion in the catalytic site together with ATP.

Introduction

Vanadate behaves in many respects like a structural analogue of inorganic phosphate [1,2] which reacts with the sarcoplasmic reticulum Ca^{2+}-ATPase in the E_2 (Ca^{2+}-depleted) conformation of the enzyme [3,4]. With the aid of vanadate it is possible to probe into the nature of intermediates of membranous and detergent solubilized Ca^{2+}-ATPase [5]. However, a quantitative study of the interaction requires information on the effect of the substrates, Ca^{2+} and ATP, on the enzyme-vanadate complex. Ca^{2+} destabilizes the complex by forcing the Ca^{2+}-ATPase into the E_1 state [6,7] and ATP has been reported to interfere with the formation of the complex by binding as a competitive inhibitor at the catalytic site [4]. During our study of the vanadate-reacted Ca^{2+}-ATPase we observed that the effect of Ca^{2+} was retarded by ATP after transfer to vanadate free media used for measurements of enzyme activity. Since such an effect was not expected on the basis of current views on the enzyme-vanadate interaction, we have undertaken an analysis of the phenomenon. This has been done by studying the interplay of Ca^{2+}, Mg^{2+}, and ATP on the release of vanadate from the vanadate-reacted Ca^{2+}-ATPase. Our results indicate that ATP locks the enzyme-vanadate complex into a much more stable state, not consistent with a purely competitive type of interaction. The effect of Mg^{2+} on the formation of the ternary

Abbreviations: E, Ca^{2+}-ATPase; EMgV, vanadate-reacted Ca^{2+}-ATPase (i.e. complex between the enzyme, vanadate and Mg^{2+}); K_d, dissociation constant; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; Tes, 2-([2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino)ethanesulphonic acid.

complex of Ca^{2+}-ATPase, vanadate and ATP suggests that the same Mg^{2+} is involved in simultaneous binding of ATP and vanadate at the catalytic site, and that the presence of an additional magnesium ion at this site destabilizes the complex.

Materials and Methods

Materials. Sarcoplasmic reticulum vesicles were prepared from rabbit skeletal muscle according to the method of De Meis and Hasselbach [8] and extracted with a low concentration of deoxycholate [9] to obtain purified Ca^{2+}-ATPase membranes. Radioactively labelled vanadyl chloride ($^{48}VOCl_2$) in 1 M HCl was obtained from the Radiochemical Centre (Amersham, Bucks., U.K.) and converted into vanadate by air oxidation after alkalinization ($\simeq$ 0.1 M excess base) with NaOH. Stock solutions of orthovanadate (100 mM) were prepared from sodium metavanadate (Merck AG, Darmstadt, F.R.G.) by addition of an excess NaOH (slightly more than two equivalents). [U-^{14}C]Adenosine 5'-triphosphate was also obtained from Amersham. (−)-Norepinephrine hydrochloride and pyruvate kinase (500 units/mg) were from Sigma Chem. Co. Unless when otherwise noted. EMgV was prepared by reacting 0.5–1 mg Ca^{2+}-ATPase/ml with vanadate at 20°C for a minimum of 0.5 h in a medium of the following composition: 1 mM Mg^{2+}, 1 mM EGTA, 10 mM Tes (pH 7.5), 100 mM KCl and 5–100 μM vanadate (with or without ^{48}V-labelled vanadate). The other reagents were analytical grade and were obtained from sources as previously described [10].

Vanadate release experiments. Release of vanadate from EMgV was initiated by 100-fold dilution of vanadate-reacted Ca^{2+}-ATPase into a medium, containing 0.01 M Tes (pH 7.5), 0.1 M KCl together with different combinations of Ca^{2+}, Mg^{2+} and/or ATP as indicated in the legends of the figures. To obtain Mg^{2+} and/or Ca^{2+} free media 1 mM EDTA or EGTA was added to 0.01 M Tes (pH 7.5) and 0.1 M KCl. In the presence of Mg^{2+} and ATP the medium was supplemented with an ATP regenerating system (1 mM phospho*enol*pyruvate and 0.025 mg pyruvate kinase per ml) and with norepinephrine (0.5 mM). The latter reagent did not affect the release process and was added to prevent recombination of vanadate and enzyme by converting released vanadate to reduced forms [11]. (By contrast attempts to block the reaction of [^{48}V]vanadate with Ca^{2+}-ATPase by addition of excess unlabelled vanadate (1 mM) strongly decreased the vanadate release rate). Release of vanadate as a function of time after dilution was measured either by determination of remaining bound ^{48}V by Millipore filtration or, alternatively, by following the recovery of Ca^{2+}-ATPase activity.

Measurement of [^{48}V] binding. In the Millipore method a sample containing 0.2 mg protein was filtered through a double filter of 0.45 μm HAWP and rinsed with 10 ml ice-cold buffer solution not containing vanadate. The radioactive content of filters and standards was measured by γ-scintillation counting. The radioactive content of the lower filter (amounting to maximally 10% of that of the upper filter) was used to correct for contaminating, unbound vanadate.

In a few experiments vanadate binding was measured after separation of bound and unbound vanadate by gel chromatography: Ca^{2+}-ATPase (1 mg) was reacted for 0.5 h at 20°C with vanadate in the usual reaction medium. The sample was then cooled to 0°C for 5 min before application to a Sephadex G-50 column (1 × 25 cm), equilibrated with buffer without vanadate at 0°C. Fractions of 1 ml were collected and analyzed for radioactivity and protein to calculate the amount of vanadate bound to protein.

Ca^{2+}-ATPase activity. Ca^{2+}-ATPase activity was measured spectrophotometrically at 20°C by a NADH-coupled reaction in the presence of an ATP regenerating system [10]. The reaction medium contained in a total volume of 2.8 ml: 0.1–0.3 mM Ca^{2+} and 0–0.2 mM EGTA (to give an excess concentration of Ca^{2+} of 0.1 mM after addition of the protein sample), 5 mM ATP, 1 mM phospho*enol*pyruvate, 6 mM Mg^{2+}, pyruvate kinase, NADH and lactate dehydrogenase as previously described [10]. The reaction was started by addition of Ca^{2+}-ATPase, involving a 100–200-fold dilution of the original vanadate/enzyme mixture. Under these conditions equilibrium binding of vanadate is negligibly low, but the release of vanadate is sufficiently slow (1–2 h^{-1}, see Results)

to permit measurement of the ATPase activity before any reactivation has occurred (cf. Fig. 2a). Reversibility of the inhibition of Ca^{2+}-ATPase by vanadate was checked by addition of Ca^{2+} to samples to obtain a concentration of 10^{-4} M Ca^{2+} or after incubation of the diluted sample at 37°C for 1 h, before measurement of enzyme activity.

Binding of ATP. Binding isotherms for ATP at 20°C were obtained by filtration of solutions containing 0.2 mg protein and 0.8–23 μM ^{14}C-labelled nucleotide through a double layer of Millipore 0.45 μm HAWP filter. The radioactive content of the filters was measured after transfer to counting vials (without rinsing) and mixing with 5 ml Lumagel (Lumac/3M B.V., The Netherlands). The radioactivity on the lower filter, which amounted to 35–70% that of the upper filter, was subtracted. Control experiments in which 5 mM unlabelled MgATP was added to the sample containing [^{14}C]ATP showed the same number of counts on the upper and lower filter, indicating the same degree of wetting of the two filters (approx. 35 μl) by this method.

Calculations. For calculation of free Ca^{2+} concentration the following apparent stability constants at pH 7.5, 0.1 M KCl and 20°C were used [10]: MgATP, 16200 M^{-1}; CaATP, 7600 M^{-1}; MgEGTA, 170 M^{-1}. A revised value [12] of $2.5 \cdot 10^7$ M^{-1} was used for CaEGTA. The ATP effect on vanadate release (Fig. 4) was analyzed by a 'Penzer plot' according to Equation 1 [13]:

$$\frac{1}{k_0 - k} = \frac{1}{k_0 - k_\infty} + \frac{1}{k_0 - k_\infty} \cdot \frac{K_d}{[ATP]} \qquad (1)$$

where k is the rate constant of vanadate release at the ATP concentration studied, k_0 is the rate constant in absence of ATP, k_∞ is the rate constant at full saturation with ATP and K_d is the dissociation constant for the complex of ATP with EMgV.

Results

Relation between vanadate binding and Ca^{2+}-ATPase activity

Fig. 1 demonstrates that [^{48}V]vanadate interacts with Ca^{2+}-ATPase at a single site and that

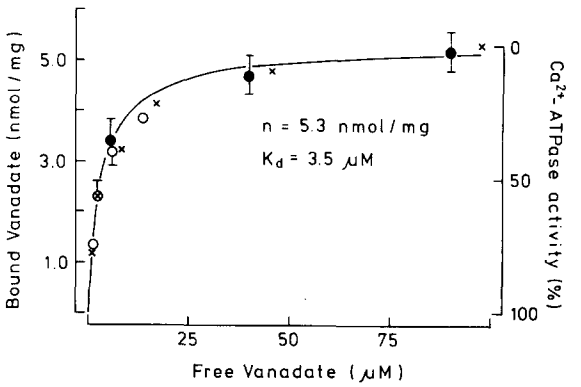

Fig. 1. Relation between vanadate binding and Ca^{2+}-ATPase activity. Ca^{2+}-ATPase was reacted for 30 min with various concentrations of [^{48}V]vanadate (5–100 μM) in the presence of 1 mM Mg^{2+}, 1 mM EGTA, 0.01 M Tes (pH 7.5) and 0.1 M KCl (Materials and Methods), and was then assayed for ^{48}V binding (○, ●) and Ca^{2+}-ATPase activity (×). (○), Millipore filtration; ●, gel chromatography. The data are fitted by a binding isotherm (drawn curve) corresponding to a single site with the indicated binding capacity (n) and dissociation constant (K_d). Bars show the S.D. of four experiments.

this interaction is followed by a corresponding decrease in ATPase activity. The binding capacity is close to the maximal capacity of our preparations for phosphorylation with ATP (4–5 nmol/mg, cf. Ref. 14). These observations are in agreement with the view that vanadate substitutes for phosphate at the catalytic site [3,4].

Effect of Ca^{2+} and ATP on vanadate release

In Fig. 1 the ATPase activity was measured after dilution of EMgV directly into the enzyme assay media. Fig. 2 demonstrates that reactivation of Ca^{2+}-ATPase activity due to the release of vanadate from the enzyme, is slow under these conditions. This is indicated by the slow acceleration of NADH oxidation as a function of time (Fig. 2 trace a, rate constant 1–2 h^{-1}). If however, the sample is first transferred to a medium containing the same Ca^{2+} and Mg^{2+} concentration as the assay medium, but no ATP, reactivation is rapid, being completed within 30 s (Fig. 2, trace b). These findings suggest that ATP binds to the vanadate-reacted enzyme and thereby inhibits release of vanadate. Fig. 3 shows that the rate constant of reactivation of EMgV is strongly dependent on the medium concentration of Ca^{2+} in

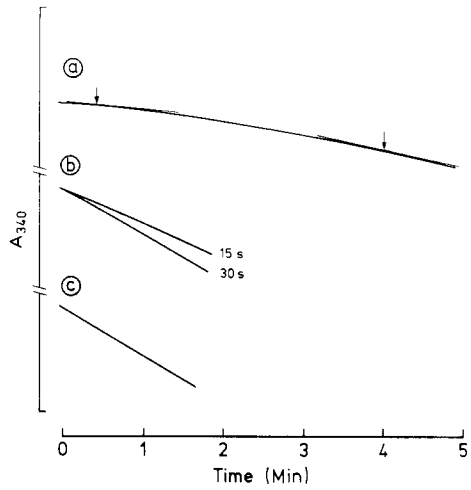

Fig. 2. Enzymatic reactivation of vanadate-reacted Ca^{2+}-ATPase. Trace a, Ca^{2+}-ATPase (1 mg/ml) was reacted with vanadate (50 μM) for 30 min, in presence of 1 mM Mg^{2+}, 1 mM EGTA, 0.01 M Tes (pH 7.5) and 0.1 M KCl, and then for spectrophotometric assay of ATPase activity 20 μl was transferred to 2.8 ml medium containing 0.1 mM Ca^{2+}, 6 mM $MgCl_2$, 5 mM ATP and other additions as described in Materials and Methods. The decrease in absorption at 340 nm (ordinate scale) represents oxidation of NADH in the coupled assay. The slope of the tangents at the arrows measures Ca^{2+}-ATPase activity. In trace b 25 μl of vanadate-treated enzyme was first diluted with 2.5 ml 0.1 mM Ca^{2+}, 1 mM Mg^{2+}, 0.01 M Tes (pH 7.5) and 0.1 M KCl. After 15 s or 30 s, the ATPase activity of 2 ml of this solution was assayed by transfer to 0.8 ml buffer containing ATP, Mg^{2+} and other additions to produce the same final concentrations as in trace a. Trace c, control experiment with Ca^{2+}-ATPase incubated in absence of vanadate but otherwise processed as for trace a.

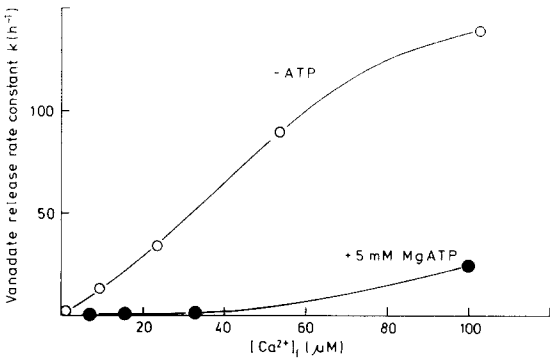

Fig. 3. Effect of Ca^{2+} on vanadate release in presence and absence of ATP. An aliquot of Ca^{2+}-ATPase (1 mg protein/ml) was incubated with 50 μM vanadate in the presence of 1 mM Mg^{2+}, 1 mM EGTA, 0.01 M Tes (pH 7.5), and 0.1 M KCl for 30 min. Portions of the aliquot were then diluted 200-fold with media containing various concentrations of Ca^{2+} (0.196–0.3 mM), 1 mM Mg^{2+}, 0.2 mM EGTA, 0.01 Tes (pH 7.5), 0.1 M KCl, 1 mM phospho*enol*pyruvate and 0.025 mg pyruvate kinase/ml with (●) or without (○) 5 mM MgATP for various periods of time before measurement of ATPase activity. The reactivation rate constants were calculated from semi logarithmic plots of inactivation versus time. The conditions routinely used for the ATPase assay (cf. Fig. 2) correspond to 35 μM free Ca^{2+}.

agreement with previous observations [6]. In the absence of ATP Ca^{2+}-stimulation of reactivation occurs between 10^{-5} M and 10^{-4} M free Ca^{2+}, suggesting a lower affinity of the vanadate-reacted Ca^{2+}-ATPase for Ca^{2+} than the affinity of the free enzyme [12]. The Ca^{2+} activation profile is consistent with a cooperative binding process (Hill coefficient approx. 1.6 for the data of Fig. 3) leading to release of vanadate. This indicates that the destabilization of the enzyme-vanadate complex induced by Ca^{2+} binding is related to the conformational transition from E_2 to E_1, which is tightly coupled with cooperative binding of Ca^{2+} [12]. The presence of ATP (5 mM) in the release media significantly retards vanadate release. The effect of ATP on the reactivation rate constant is most pronounced at the lower Ca^{2+} concentrations, suggesting that the retarding effect of nucleotide on vanadate release is exerted mainly in the E_2 state of the enzyme.

Effect of ATP on vanadate release in absence of Ca^{2+} and Mg^{2+}

Fig. 4 shows [^{48}V]vanadate release from EMgV after dilution into EDTA media, containing various concentrations of ATP. It is seen that even 1–2 μM ATP delays vanadate release appreciably and that release is decreased almost to zero above 50 μM ATP. The data can be accounted for by binding of ATP with high affinity to EMgV (Penzer plot, inset of Fig. 4, $K_d = 4$ μM), assuming the rate of vanadate release to be infinitely low at full saturation with ATP. High affinity binding is directly confirmed by binding measurements with [^{14}C]ATP under the same conditions (Fig. 5). EMgV binds one mole ATP per mole active enzyme with a dissociation constant of 4.3 μM. Fig. 5 also shows that the affinity of EMgV is significantly higher than that of the vanadate unreacted enzyme ($K_d = 22$ μM). This relatively low affinity

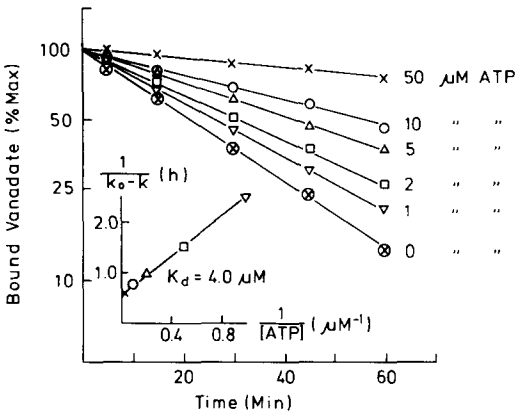

Fig. 4. Effect of ATP on vanadate release. An aliquot of Ca^{2+}-ATPase (1 mg/ml) was reacted with 15 μM vanadate in the presence of 1 mM Mg^{2+}, 1 mM EGTA, 0.01 M Tes (pH 7.5) and 0.1 M KCl, and then diluted 100-fold with 1 mM EDTA, 0.01 M Tes (pH 7.5) and 0.1 M KCl, containing various concentrations of ATP as specified. After the indicated time intervals ^{48}V binding to Ca^{2+}-ATPase was measured by the Millipore filtration procedure. The inset shows a Penzer plot of the data according to Eqn. 1 given in Materials and Methods with the resulting K_d for ATP binding indicated.

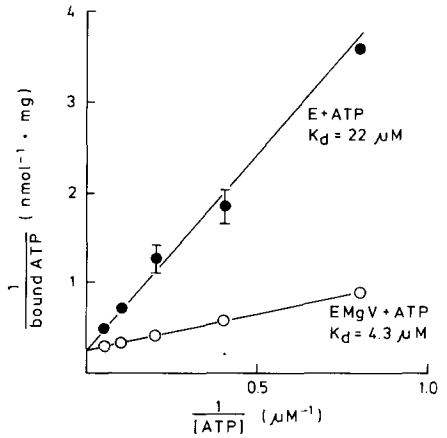

Fig. 5. Analysis of binding [^{14}C]ATP to vanadylated and vanadate unreacted Ca^{2+}-ATPase in absence of Mg^{2+}. Ca^{2+}-ATPase (1 mg/ml) was incubated for 30 min with (○) or without (●) 100 μM vanadate in the presence of 1 mM Mg^{2+}, 1 mM EGTA, 0.01 M Tes (pH 7.5), 0.1 M KCl, before dilution of 0.2 mg protein 100-fold with 1 mM EDTA, 0.01 M Tes (pH 7.5), 0.1 M KCl and [^{14}C]ATP (0.8–23 μM). Binding of [^{14}C]ATP was measured after Millipore filtration of the whole sample as described in Materials and Methods. The lines correspond to a binding capacity of $n = 5.0$ nmol/mg and the dissociation constants shown. Each point indicates the average of four determinations. S.D. is shown, when larger than the size of the symbols.

for uncomplexed ATP in absence of vanadate is in agreement with previous findings [10,15] and contrasts with the high affinity of Ca^{2+}-ATPase for MgATP ($K_d = 2.7$ μM, see below).

Effect of Mg^{2+} plus ATP on vanadate release

In the absence of ATP addition of Mg^{2+} (1–10 mM) together with EGTA to the release media, instead of EDTA, did not influence the rate of vanadate release (data not shown). However, the presence of Mg^{2+} has a dramatic effect on the ATP concentration dependence of vanadate release. Fig. 6 shows Penzer plots of data obtained at 1 mM and 10 mM free Mg^{2+}. This analysis indicates that under these conditions K_d for the ATP effect is increased to 130 μM and 810 μM, respectively, representing a 32- and 203-fold decrease in apparent affinity below that observed in EDTA. These data suggest that MgATP is bound with much less affinity by EMgV than uncomplexed ATP. Supplementing binding experiments with [^{14}C]ATP in the presence of 1 or 10 mM

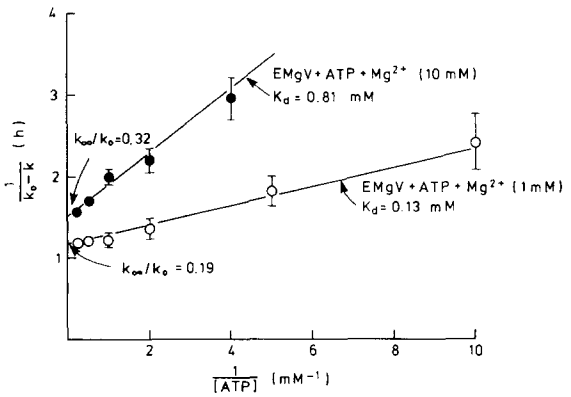

Fig. 6. Penzer plots of the effect of ATP on vanadate release in the presence of Mg^{2+}. The experiments were performed as described in the legend to Fig. 4, except that the dilution medium instead of 1 mM EDTA contained 1 mM (○) or 10 mM (●) free Mg^{2+} together with 1 mM EGTA, and was supplemented with 1 mM phospho*enol*pyruvate, 0.025 mg pyruvate kinase/ml and 0.5 mM norepinephrine to reduce released vanadate to vanadyl ions. The ATP concentration was varied between 0.1 and 5 mM. Each point indicates the average of three determinations. S.D. is shown, when larger than the size of the symbols. The apparent dissociation constant for the ATP effect (K_d) and rate constant for vanadate release at an infinite (saturating) concentration of ATP (k_∞) relative to that observed in the absence of ATP (k_0), are indicated.

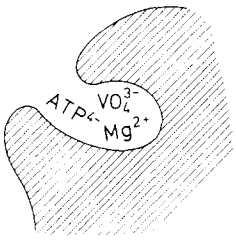

Fig. 7. Model of the catalytic site of Ca^{2+}-ATPase proposed on basis of the present results.

Mg^{2+} showed that after reaction of Ca^{2+}-ATPase with vanadate the high-affinity binding of MgATP observed in the absence of vanadate ($K_d = 2.7$ μM) is reduced to a negligible level (which could not be measured accurately by the present technique). This raises the question, whether the apparent low affinity effect of MgATP on vanadate release could be accounted for solely by the presence of a small fraction of ATP in the uncomplexed form, which would be expected to bind with the same affinity as measured in the presence of EDTA ($K_d = 4$ μM). At 1 and 10 mM Mg^{2+} calculation based on the stability constant cited in Materials and Methods shows that approx. 4% and 0.4%, respectively, of the total ATP is in the uncomplexed form. This would correspond to a 25- and 250-fold decrease in apparent affinity for the ATP effect, if it is assumed that MgATP does not inhibit vanadate release. These numbers are in fairly good agreement with those actually observed. However, the data of Fig. 6 shows that the situation is more complex. The rate constant for vanadate release at saturating ATP concentration (k_∞) increases with increasing Mg^{2+} concentration in contrast to the release measured in absence of ATP (k_0) which is independent of Mg^{2+} as stated above. This suggests that MgATP binds with low affinity to EMgV and thereby destabilizes the resulting complex relative to the complex obtained when metal-free ATP is bound to EMgV.

Discussion

The present data show that ATP and vanadate can bind simultaneously to Ca^{2+}-ATPase with high affinity. Furthermore ATP decreases the rate of vanadate release from EMgV after dilution with vanadate free media, to a vanishingly small value. This effect of ATP occurs especially in the absence of free Mg^{2+} and is opposite to the effect of Ca^{2+}, which by binding to EMgV causes a destabilization of the enzyme-vanadate complex (Fig. 3), presumably by forcing the enzyme from the E_2 to E_1 state. Previous studies have suggested that ATP lowers the activation energy for the $E_2 \leftrightarrow E_1$ transition in the vanadate-free state, causing an acceleration on the conversion of E_2 to E_1 [16,17]. It is evident that the delay in vanadate release observed here is exerted via an entirely different mechanism. We suggest that ATP by binding to the catalytic site, because of proximity effects, acts as a 'lock' in the release of vanadate from the enzyme. This implies that the catalytic site at least in the E_2 state is large enough to accommodate both ATP and vanadate (or, by analogy, ATP and phosphate). The suggestion of proximity of ATP and vanadate on the enzyme is supported by the demonstration of high affinity of EMgV for ATP in Mg^{2+} free media (Fig. 5) suggesting that the affinity of uncomplexed ATP is increased by interaction with the same Mg^{2+} that is bound in EMgV at the catalytic site. These considerations have been summarized in Fig. 7, which shows a model of the catalytic site containing vanadate and ATP, both interacting with the same magnesium ion.

From previous studies on the reversal of the phosphorylation reaction there is precedence for the view that Mg^{2+} may mediate contact between two ligands: Formation of MgATP from ADP-sensitive phosphoenzyme has been shown to require uncomplexed ADP (EMgP + ADP → E + MgATP) [18]. Accordingly the same Mg^{2+} is probably shared between the phosphate group and ADP in the transition state (EMg(P)ADP).

In the presence of Mg^{2+}, EMgV may react with ATP, Mg^{2+} and MgATP to form EMgVATP or EMgVMgATP. The latter complex is much less stable than the former. This is evident from the lower apparent affinity of EMgV for MgATP than for ATP and from the increase in vanadate release rate at saturating ATP concentrations (k_∞) induced by Mg^{2+} (Fig. 6). The destabilization may be caused by unfavourable electrostatic and/or steric interactions between the two Mg^{2+} simultaneously present at the catalytic site. It is also possible that MgATP destabilizes EMgV by bind-

ing at another nucleotide site with low affinity [10].

Our findings are relevant in relation to the modulatory effect of ATP on Ca^{2+}-ATPase activity. The complex non-linear dependence in Lineweaver-Burk plots of Ca^{2+}-ATPase on MgATP concentration has been analyzed in terms of a high-affinity and a low-affinity ATP binding site [10,19,20]. Binding of modulatory ATP to the catalytic site with different apparent affinities in the phosphorylated and non-phosphorylated state may be an important factor to account for the high-affinity and low-affinity dependence [21]. The present observations suggest that metalfree ATP may bind with high affinity to phosphorylated Ca^{2+}-ATPase in analogy with the situation for the vanadate-reacted enzyme. In the presence of millimolar Mg^{2+} the apparent affinity probably would be low due to the high percentage of ATP present in the Mg^{2+} complexed form. In addition MgATP may be bound with low affinity to phosphorylated enzyme. It is likely that destabilization of the phosphoenzyme and increased turnover results from binding of MgATP rather than ATP in analogy with the destabilization of EMgV induced by MgATP.

Acknowledgments

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