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FACTORS AFFECTING THE INHIBITION OF YEAST PLASMA MEMBRANE ATPase BY VANADATE

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Summary

Inhibition of yeast plasma membrane ATPase by vanadate occurs only if either Mg^{2+} or $MgATP^{2-}$ is bound to the enzyme. The dissociation constant of the complex of vanadate and inhibitory sites is 0.14—0.20 μM in the presence of optimal concentrations of Mg^{2+} and of the order of 1 μM if the enzyme is saturated with $MgATP^{2-}$. The dissociation constants of Mg^{2+} and $MgATP^{2-}$ for the sites involved are 0.4 and 0.62—0.73 mM, respectively, at pH 7.

KCl does not increase the affinity of vanadate to the inhibitory sites as was found with ($Na^+ + K^+$)-ATPase. On the other hand, the effect of Mg^{2+} upon vanadate binding is similar to that upon ($Na^+ + K^+$)-ATPase, and the corresponding affinity constants of Mg^{2+} and vanadate for the two enzymes are of the same order of magnitude.

Introduction

Vanadate is a potent inhibitor of ($Na^+ + K^+$)-ATPase (ATP phosphohydrolase, EC 3.6.1.3) [1—3]. The inhibition is maximal in the presence of high concentrations of Mg^{2+} and K^+ [1,4]. Yeast plasma membrane ATPase is also inhibited by vanadate [5]. The inhibition is of the non-competitive type [6]. We have now examined whether the inhibition of yeast plasma membrane ATPase by vanadate shows a dependence upon the Mg^{2+} concentration and the K^+ concentration similar to ($Na^+ + K^+$)-ATPase.

Abbreviation: Mes, 4-morpholineethanesulfonic acid.

Methods

Yeast plasma membranes were isolated from the yeast, *Saccharomyces cerevisiae* Delft 2, as described in Ref. 7. The ATPase activity was determined at 30°C in a 25 mM Tris/25 mM Mes buffer, with 4 mM Na_2ATP as substrate, unless otherwise stated. In some experiments, 50 mM KCl was added. The orthophosphate liberated was determined as described in Ref. 7. Mg^{2+} was added as $MgSO_4$. In addition to the Mg^{2+} added, some Mg^{2+} was also present as a contaminant in the buffer and in the ATP. The total concentration of Mg^{2+} present as contaminant was determined by atomic absorption. Concentrations of free Mg^{2+} and of $MgATP^{2-}$ were calculated according to the method described in Ref. 8. We accounted for changes in the ionic strength and also for the formation of complexes between Mg^{2+} and SO_4^{2-} [9]. In addition, we made appropriate corrections for the decrease in ATP concentration during the incubation by taking the mean ATP concentration instead of the initial ATP concentration.

The disodium salt of vanadate-free ATP was obtained both from Boehringer, Mannheim, F.R.G., and from Sigma, St. Louis, U.S.A. (grade II). ATP containing vanadate (equine muscle ATP) was obtained from Sigma.

The acid dissociation constant of monovalent vanadate was determined at 24°C by measuring the extinction of 0.1 mM vanadate at 266 nm at varying pH in 50 mM Tris/50 mM Mes buffer both in the presence and in the absence of varying $MgSO_4$ concentrations.

Results

Fig. 1 shows that the pH dependence of yeast plasma membrane ATPase was affected by the presence of vanadate in the ATP sample used. With a 4 mM ATP solution of equine muscle ATP from Sigma the vanadate concentration was approx. 1 μM. The relative decrease in ATPase activity was maximal at pH 6.7. Half-maximal inhibition was found at both pH 6.15 and 8.15.

Fig. 2 shows that the ATPase activity increased on increasing the Mg^{2+} concentration up to 2 mM Mg^{2+} in the absence of added vanadate. At higher Mg^{2+} concentrations a subsequent decrease in ATPase activity was found.

The inhibition (I), expressed in percentage points based on the rate of orthophosphate liberation found in the absence of vanadate, increased with the vanadate concentration. The concentration at which this inhibition was half-maximal depended both upon the pH and upon the concentration of added Mg^{2+}. At pH 7 maximal inhibition was attained at a total Mg^{2+} concentration of 5 mM and higher, up to 30 mM (Fig. 3). At pH 8.5, however, increasing the total Mg^{2+} concentration above 5 mM led to a decrease in the sensitivity of the ATPase to vanadate. In addition, at all concentrations of Mg^{2+} examined the sensitivity to vanadate was lower at pH 8.5 than at pH 7.

The dependence of the inhibition of ATPase activity (I) upon the concentration of vanadate (V) could be described by a Michaelis-Menten equation:

$$I = 100\left(1 - \frac{v_+}{v_-}\right) = \frac{I_{max}\,V}{K_V + V} = I_{max} - K_V(I/V) \qquad (1)$$

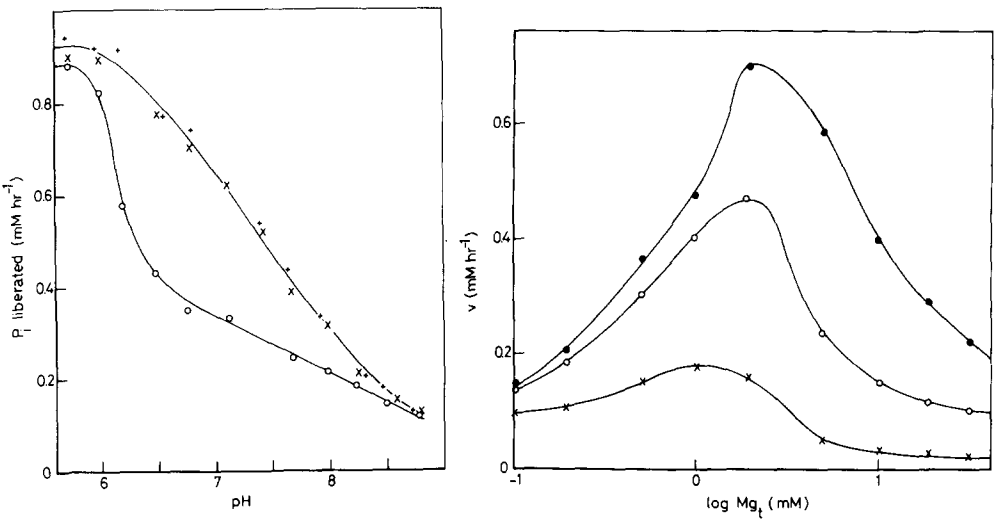

Fig. 1. Effect of the pH upon the ATPase activity found both in the presence and in the absence of vanadate. +, vanadate-free ATP from Boehringer; X, vanadate-free ATP from Sigma; ○, ATP containing vanadate from Sigma. Concentration of ATP and of $MgSO_4$: 4 mM. Incubation time: 60 min.

Fig. 2. Dependence of the ATPase activity upon the concentration of added Mg^{2+} at varying concentrations of vanadate. ●, no vanadate; ○, 0.4 μM vanadate; X, 5 μM vanadate. 4 mM ATP at pH 7. Incubation time: 60 min. In this and all further experiments, vanadate-free ATP from Boehringer was used.

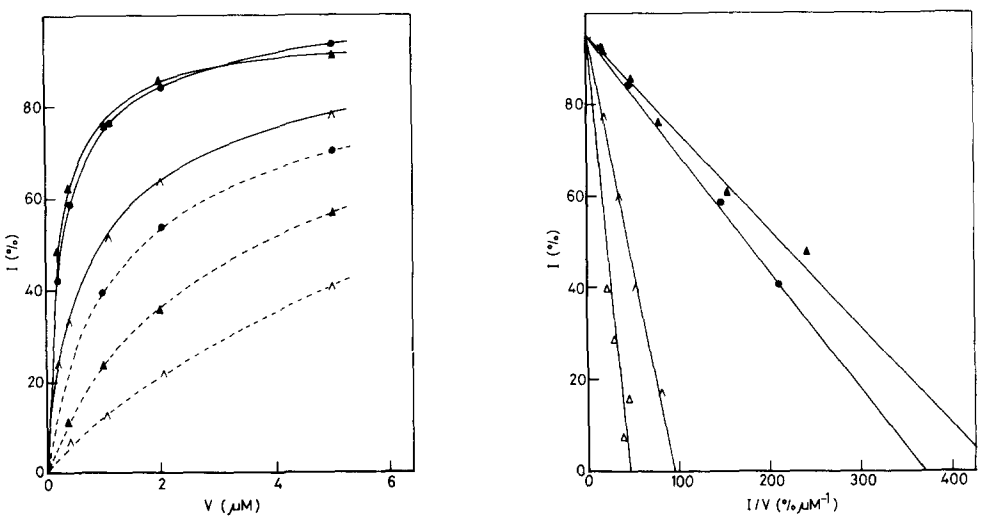

Fig. 3. Dependence of the inhibition of the ATPase activity upon the concentration of added vanadate (v) at varying concentrations of Mg^{2+} and at two pH values. (∧) 2, (●) 5 and (▲) 20 mM total Mg^{2+}. (———) pH 7, (- - - - -) pH 8.5. The inhibition (I) is expressed in percentage points based on the control value without vanadate. In this experiment and all further experiments the amount of orthophosphate liberated between 5 and 65 min incubation was taken as a measure for the rate of orthophosphate liberation, since the inhibition was not immediately maximal, but developed within approx. 5 min.

Fig. 4. Plot of the inhibition (I) against the quotient of this inhibition and the vanadate concentration. (△) 0.5, (∧) 2, (●) 5 and (▲) 20 mM total Mg^{2+} at pH 7.0; see also legend to Fig. 3.

A straight line was found on plotting I against I/V as seen in Fig. 4. Similar plots were found at other pH values and at varying concentrations of Mg^{2+}. The maximal inhibition varied from 85 to 95%.

At pH 7 and lower the dissociation constant of the complex of vanadate and the inhibitory sites decreased with increasing concentration of free Mg^{2+} (see Fig. 5). At pH 8 and higher, however, increasing the free Mg^{2+} concentration above 1 mM led to a subsequent increase in the dissociation constant.

We have considered the possibility that the increase in K_V found at high pH and at high Mg^{2+} concentrations was due to complex formation by Mg^{2+} and divalent vanadate which would cause the concentration of available vanadate to decrease. The association constant of the complex of divalent vanadate and Mg^{2+} (K_{ass}) was calculated from the values of the apparent acid dissociation constant of monovalent vanadate found at varying Mg^{2+} concentrations (K_{app}) according to Eqn. 2. It was assumed that complex formation of Mg^{2+} and monovalent vanadate could be neglected.

$$K_{app} = K_d f_{V,1} f_{V,2}^{-1}(1 + Mg f_{Mg} f_{V,2} K_{ass}) \quad (2)$$

K_d is the thermodynamic dissociation constant of monovalent vanadate (see Table I). The value of pK_d (8.84) was somewhat higher than the value of pK_d (8.3) calculated from the data of apparent dissociation constants reported in Ref. 10. $f_{V,1}$, $f_{V,2}$ and f_{Mg} are the activity coefficients of monovalent vanadate, divalent vanadate and Mg^{2+}, respectively. These values were calculated as described in Ref. 11. Mg is the concentration of free Mg^{2+}. Table I shows that except at the highest Mg^{2+} concentration applied the corrected

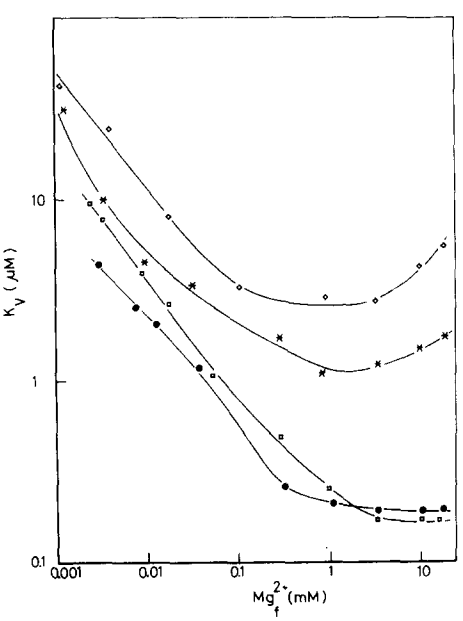

Fig. 5. Plot of the dissociation constant of vanadate (K_V) against the concentration of free Mg^{2+} at varying pH values. (□) pH 6.8, (●) pH 7.0, (☆) pH 8.0 and (◇) pH 8.5. ATP concentration 4 mM.

TABLE I

VALUES OF K_V FOUND AT HIGH Mg^{2+} CONCENTRATIONS IN THE ABSENCE OF KCl AND CORRECTIONS FOR DISSOCIATION OF MONOVALENT VANADATE TO DIVALENT VANADATE AND COMPLEXATION OF DIVALENT VANADATE WITH Mg^{2+}

K_V and K'_V are the dissociation constants of vanadate before and after correction for the presence of divalent vanadate and divalent vanadate-Mg^{2+} complexes, respectively. K'_V refers to monovalent vanadate only. I is the ionic strength. The mean error was about 14% of the value concerned. pK_d = 8.84 ± 0.02 (S.E.) (four determinations), pK_{ass} = 1.96 at 24°C. Corrections for the difference in temperature at which the ATPase activity was determined were made on assuming that the effect of the temperature upon the dissociation constant of monovalent vanadate and upon the association constant of divalent vanadate and Mg^{2+} was equal to the effect of the temperature upon the corresponding constants for orthophosphate [9,12].

Total Mg^{2+} (mM)	Free Mg^{2+} (mM)	K_V (pH 8.4) (μM)	K'_V (μM)	K_V (pH 8.0) (μM)	K'_V (μM)	I (mM)
5	1.1	2.82	1.61	0.91	0.68	56
10	4.5	3.43	1.84	0.90	0.66	65
20	11	4.01	1.93	1.21	0.84	92
30	17	6.32	2.80	1.43	0.96	116

values of K_V (K'_V) found at the varying Mg^{2+} concentrations did not differ greatly both at pH 8.4 and at pH 8.0.

It appeared that the minimal value of K'_V found at optimal Mg^{2+} concentrations was related to the proton activity (H) by:

$$K'_{V,min} = K'_{V,min,o}(1 + K_H/H) \quad (3)$$

The kinetic constants (see Table II) differed only slightly from those found in the presence of 50 mM KCl.

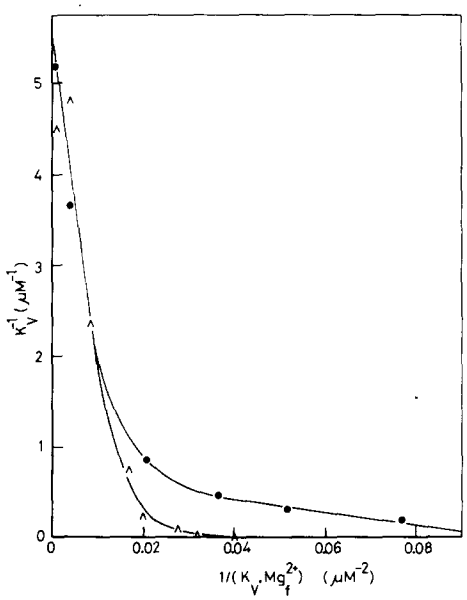

Fig. 6. Plot of the reciprocal value of the dissociation constant of vanadate against the quotient of the reciprocal constant and the concentration of free Mg^{2+} found at pH 7. (•) 4 mM ATP, (∧) 1 mM ATP.

If only one Mg^{2+} binding site were involved in the enhancement of the inhibition of membrane ATPase by Mg^{2+}, a linear relationship would be expected according to Eqn. 4.

$$K_V^{-1} = K_{V,min}^{-1} Mg/(K_{Mg} + Mg) = K_{V,min}^{-1} - K_{Mg}(K_V^{-1}/Mg) \quad (4)$$

$K_{V,min}$ is the value obtained at infinitely high Mg^{2+} concentrations, and K_{Mg} is the affinity constant of Mg^{2+} for the Mg^{2+} binding sites.

At pH 7 and also at other pH values (data not shown), however, a concave curve was found (see Fig. 6). The upper part of the curve appeared to be independent of the concentration of ATP added, whereas the lower part of the curve decreased considerably on decreasing the ATP concentration from 4 to 1mM.

Concave curves like these indicate that two binding sites for Mg^{2+} are involved [12]: a low-affinity site giving rise to the upper part of the curve and a high-affinity site giving rise to the lower part of the curve. Approximate values of the dissociation constants of Mg^{2+} from the complexes of Mg^{2+} with the binding sites were obtained from the slopes to the curves (see Table II). Addition of 50 mM KCl did not affect the affinity of Mg^{2+} to the Mg^{2+} binding sites at pH 7.0. The affinity of Mg^{2+} decreased on lowering the pH. The latter could be demonstrated only in the presence of KCl, since at low pH and in the absence of added KCl, the enzyme is not stable [7].

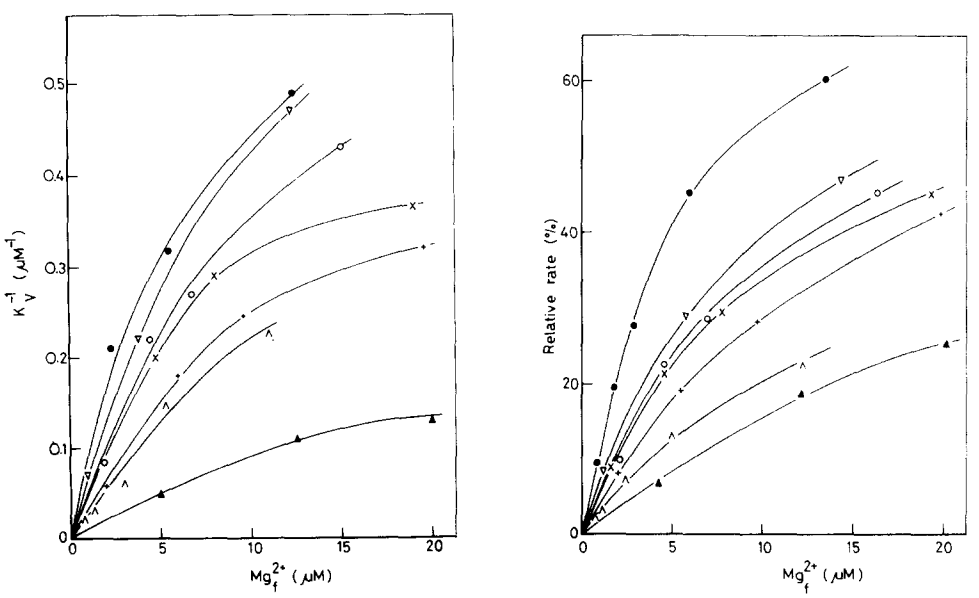

Fig. 7. Dependence of the reciprocal value of the dissociation constant of vanadate upon the concentration of free Mg^{2+} at relatively low Mg^{2+} concentrations. Without KCl: (●) pH 7.0, 4 mM ATP; (^) pH 7.0, 1 mM ATP. With 50 mM KCl and 4 mM ATP: (▽) pH 7.5, (○) pH 7.0, (X) pH 6.75, (+) pH 6.5, (▲) pH 6.0.

Fig. 8. Dependence of the relative rate of ATP hydrolysis upon the concentration of free Mg^{2+}. Data of Fig. 7. The relative rate is the rate of ATP hydrolysis divided by the maximal rate of ATP hydrolysis extrapolated according to Eqn. 5 and multiplied by 100%. See further legend to Fig. 7.

TABLE II

DISSOCIATION CONSTANTS OF Mg^{2+}, $MgATP^{2-}$ AND VANADATE FOR THE YEAST PLASMA MEMBRANE ATPase

K_{Mg} is the dissociation constant of Mg^{2+} from the Mg^{2+} binding sites involved in the binding of vanadate. $K'_{V,min,o}$ is the dissociation constant of monovalent vanadate at low pH and at optimal Mg^{2+} concentrations. The dissociation constant of monovalent vanadate as a function of the pH is related to the proton activity according to Eqn. 3, by which equation K_H is defined. K_{MgATP} is the dissociation constant of the complex $MgATP^{2-}$ to the enzyme found at relatively low Mg^{2+} concentrations. $K_{V,MgATP}$ is the dissociation constant of vanadate found when the enzyme is saturated with $MgATP^{2-}$. n.d., not determined. Values are expressed as μM.

	—KCl	+50 mM KCl
K_{Mg} (pH 6.0)	n.d.	3000
K_{Mg} (pH 7.0)	400	400
$K'_{V,min,o}$	0.14	0.20
K_H	0.043	0.038
K_{MgATP} (pH 6—8)	730	620
$K_{V,MgATP}$ (pH 6.5—7.5)	0.84	0.84
$K_{V,MgATP}$ (pH 6.0)	n.d.	0.48

We have examined the effect of the Mg^{2+} concentration upon K_V^{-1} in the range of concentrations at which the high-affinity site was involved in more detail.

Fig. 7 shows that the dependence of the reciprocal value of the dissociation constant of vanadate upon the concentration of free Mg^{2+} was affected both by decreasing the pH and by the presence of KCl. A similar dependence was found for the relative rate of ATP hydrolysis in the absence of added vanadate (see Fig. 8). This relative rate (v_{rel}) equals the rate of hydrolysis

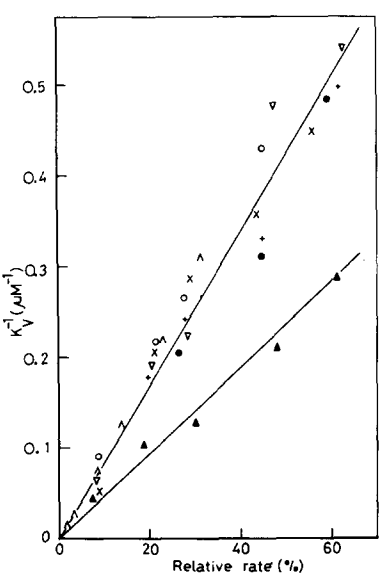

Fig. 9. Plot of the reciprocal value of the dissociation constant of vanadate against the relative rate of ATP hydrolysis. Data of Figs. 7 and 8. See also legends to Figs. 7 and 8.

of ATP multiplied by 100% and divided by the maximal rate of ATP hydrolysis expected when high concentrations of Mg^{2+} did not decrease the rate of ATP hydrolysis. The maximal rate expected was obtained from plots of the rate of ATP hydrolysis against the quotient of this rate and the concentration of the $MgATP^{2-}$ complex according to Eqn. 5.

$$v = \frac{V[\text{MgATP}]}{K_m + [\text{MgATP}]} = V - K_m(v/[\text{MgATP}]) \quad (5)$$

At relatively low concentrations of free Mg^{2+} a straight line was found enabling us to calculate V and K_m (Table II).

Fig. 9 shows that the reciprocal value of the dissociation constant of vanadate was linearly related to the relative rate of ATP hydrolysis according to Eqn. 6.

$$K_V^{-1} = K_{V,\text{MgATP}} v_{rel}/100 \quad (6)$$

By approximation, a single relationship was found at all pH values examined except at pH 6.0.

Discussion

The interaction of vanadate with yeast plasma membrane ATPase depends upon the pH, the presence of KCl, the concentration of Mg^{2+} and the concentration of ATP added. KCl does not enhance the inhibition of the ATPase by vanadate. In this respect, the yeast membrane ATPase differs from the $(Na^+ + K^+)$-ATPase [1,4].

Mg^{2+} greatly increases the affinity of vanadate for the inhibitory sites. At pH 7 the dissociation constant of Mg^{2+} from the Mg^{2+} binding site involved in the interaction of vanadate and Mg^{2+} with the ATPase amounts to about 0.4 mM in the absence of KCl. This value is of the same order of magnitude as the value found for $(Na^+ + K^+)$-ATPase at pH 7.4 but in the presence of optimal K^+ concentrations [4]. The dissociation constant of Mg^{2+} increases on lowering the pH, which may indicate that protons and Mg^{2+} compete for the same site.

When the Mg^{2+} binding site is saturated with Mg^{2+}, the dissociation constant of the vanadate inhibitory site complex amounts to 0.14—0.20 μM at pH 6. This value increases with increasing pH (see Eqn. 3). This indicates that the vanadate binding site should be in the protonated form in order to bind vanadate. The pK value of this site is 7.4. The value of the affinity constant of vanadate for the inhibitory site found in the presence of Mg^{2+} is of the same order of magnitude as that reported for $(Na^+ + K^+)$-ATPase found in the presence of optimal concentration of both K^+ and Mg^{2+} [4]. The affinity constant found by us is much lower than that reported until now for yeast ATPases [5,6] and also for *Neurospora crassa* membrane ATPase [14]. This difference can be accounted for at least in great part by the fact that the inhibition of yeast plasma membrane ATPase develops with a lag time of about 5 min. The values of the affinity constant reported were determined after relatively short incubation periods, and the inhibition of ATPase by vanadate was probably not maximal. Another factor contributing to the differences found was that optimal Mg^{2+} concentrations were not always applied.

Willsky [5] has stressed that yeast plasma membrane ATPase has more features in common with ($Na^+ + K^+$)-ATPase than with mitochondrial ATPase. Our study shows that the similarity with ($Na^+ + K^+$)-ATPase is also true as far as the affinities of both Mg^{2+} and vanadate for the inhibitory sites are concerned.

At low Mg^{2+} concentrations (less than 0.1 mM) the affinity of vanadate to the inhibitory site does not only depend upon the Mg^{2+} concentration but also upon the concentration of ATP added. In addition, the affinity depends upon the pH and the presence of KCl (see Fig. 7). Both the effect of the pH and the effect of KCl and also the effect of the total ATP concentration can be ascribed to differences in the extent of saturation of the enzyme with the $MgATP^{2-}$ complex. As shown by Ahlers et al. [15], the actual substrate of yeast plasma membrane ATPase is the complex of Mg^{2+} and ATP^{4-}: $MgATP^{2-}$ and not ATP^{4-}. The rate of hydrolysis in the absence of added vanadate will be proportional to the concentration of the enzyme $MgATP^{2-}$ complex. Fig. 9 shows that a linear relationship exists between the reciprocal dissociation constant of vanadate and the concentration of the complex of $MgATP^{2-}$ and the enzyme. This indicates that binding of $MgATP^{2-}$ to the enzyme activates the vanadate binding site. At saturation of the substrate sites with $MgATP^{2-}$, the dissociation constant of vanadate is of the order of 1 μM. Increasing the ionic strength has no significant effect upon the affinity of vanadate for the inhibiting sites.

Inhibition of yeast membrane ATPase by vanadate is of the non-competitive type [6]. This has been confirmed by us under conditions in which the concentration of free Mg^{2+} is not limiting any more [16]. It is, however, evident from the results presented in this paper that at concentration of Mg^{2+} below 0.1 mM a non-competitive type of inhibition will no longer be found, but a mixed uncompetitive-non-competitive inhibition.

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