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Electrostatic analysis of effects of ions on the inhibition of corn root plasma membrane Mg^{2+}-ATPase by the bivalent orthovanadate

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The microelectrophoretic mobility of corn root plasma membranes and the inhibition of the Mg^{+2}-ATPase by vanadate were investigated under different ionic conditions. The Mg^{2+}-ATPase was uncompetitively inhibited and a 10-fold variation of the apparent inhibition constant was observed, depending on the addition of K^+ and Mg^{2+}. The determination of the zeta potential indicated that a 5-fold decrease of the apparent inhibition constant was due to aspecific electrostatic interactions of the vanadate anion and the negative charge of the membrane. The screening and masking effects of 6 mM free Mg^{2+} totally abolished electrostatic interactions and allowed the direct determination of the intrinsic vanadate inhibition constant (K_{II}). On the other hand, a specific, non-electrostatic, effect of K^+ caused a 2-fold decrease of the inhibition constant in addition to the electrostatic effect. Finally, the electrostatic analysis indicates that the Mg^{2+}-ATPase is inhibited by the monomeric bivalent anion HVO_4^{2-}.

Introduction

Vanadate, in the +5 oxidation state, is a potent inhibitor of phosphohydrolases, which form a covalent phosphorylated intermediate [1]. Vanadate is an important criterion to identify the so-called $E_1:E_2$ ion-translocating ATPases associated with animal [2], fungal [3] and plant cell membranes [4]. Furthermore, this inhibitor has since been used for investigating mechanisms of Mg^{2+}-ATPase [5]. Unfortunately, the chemistry of vanadium solutions is exceptionally complicated [6,7]. When the total concentration of vanadate is less than approx. 100 μM and in the physiological pH range, the significant species present are the monomeric anions, $H_2VO_4^-$, and HVO_4^{2-}, and the oligomeric anion, $V_3O_9^{3-}$. Such solutions can also contain traces of the dimer, $HV_2O_7^{3-}$. There is no direct evidence of the exact form of vanadate that causes Mg^{2+}-ATPase inhibition. It has been well demonstrated that vanadate binds on the catalytic sites of various ATPases [2,8].

Vanadate binding and inhibition of the Mg^{2+}-ATPase activity are strongly enhanced in the presence of Mg^{2+} and also of K^+ in the case of animal K^+-transporting Mg^{2+}-ATPase [2,9,10]. The presence of a K^+-transporting Mg^{2+}-ATPase on the plant membrane has not been demonstrated and its existence remains controversial [11]. Nevertheless, a 3-fold decrease of the vanadate inhibition constant was observed upon addition of K^+ to the plasmalemma Mg^{2+}-ATPase of red beet [4]. In a previous paper, we have shown that the accessibility of the anion Mg^{2+}-ATP to the catalytic site is decreased due to the electrostatic repulsion of the negative surface charge of the membrane [12]. Consequently, the Michaelis constant (K_M) determined from the bulk concentration of Mg^{2+}-ATP is an apparent constant. Addition of K^+ depolarizes the membrane surface via the aspecific screening effect, as predicted by the Gouy-Chapman model, and causes a decrease in the apparent K_M, while the intrinsic constant (i.e., the constant in the absence of electrostatic interactions) is not modified. The decrease in the apparent affinity of membrane sites for various anions (NADH, anilinonaphthalenesulfonate, ATP) is quantitatively accounted for by the measured depolarization of the membrane surface and the valency of the anions via the Boltzmann relation [12–14]. The inhibition constant determined

Abbreviations: Bistris propane, 1,3-bis(tris(hydroxymethyl)methylamino)propane; Mes, 4-morpholineethanesulfonic acid.

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from the bulk concentration of vanadate is expected to be an apparent constant, and electrostatic interactions are to be taken into account for the analysis of the effects of K^+ and Mg^{2+}. Furthermore, the electrostatic analysis can be used to determine the exact inhibiting form of vanadate, since the different species in solution have different valencies.

Materials and Methods

Plant material

Corn seeds (*Zea mays* L., var Contessa) were surface-sterilized for 15 min with 3% calcium hypochlorite, rinsed in running tap water, sown on a plastic grid above an aerated solution containing 0.1 mM $CaSO_4$ and grown for 5 d in the dark. The solution was changed once.

Membrane preparation

Crude plasma membrane vesicles were prepared from excised roots as described by De Michelis and Spanswick [15] without any modification except that Bistris propane-Mes was replaced by Bistris propane-Cl. Briefly, roots were homogenized with a mortar and pestle in a grinding medium containing 25 mM Bistris propane-Cl buffer (pH 7.6)/250 mM sucrose/2 mM DL-dithiothreitol/2 mM $MgSO_4$/2 mM ATP/10% glycerol/2 mM EGTA/1 mM phenylmethylsulfonyl fluoride and 0.5% bovine serum albumin, using a medium-to-tissue ratio of 4 ml per g fresh weight. The brei was strained through cheesecloth and the homogenate was centrifuged for 10 min at $13000 \times g$. The supernatant was centrifuged for 30 min at $80000 \times g$ and the pellet (crude plasma membranes) was resuspended in a grinding medium containing 250 mM KI, incubated on ice for 15 min, and then sedimented for 30 min at $80000 \times g$. The pellet was gently resuspended in a buffer containing 2 mM Bistris propane-Cl (pH 7.0/250 mM sucrose/1 mM DL-dithiothreitol. This suspension was layered on a 30% sucrose cushion and centrifuged for 90 min at $80000 \times g$. The plasma-membrane-enriched pellet was finally resuspended in a medium containing 2 mM Bistris propane-Cl (pH 7.0)/250 mM sucrose/10% glycerol/1 mM DL-dithiothreitol/0.2% bovine serum albumin and stored in liquid N_2.

ATPase assays

The plasma membrane Mg^{2+}-ATPase activity was operationally defined as the Mg^{2+}-dependent ATP hydrolysis in the presence of molybdate, nitrate and azide (which inhibit phosphatases, tonoplastic and mitochondrial Mg-ATPases, respectively). We will show that this Mg^{2+}-ATP hydrolysis is totally vanadate-sensitive. The standard incubation medium (0.5 ml final volume) contained, unless otherwise indicated: 25 mM Tris-Mes (pH 6.5)/25 mM KNO_3/25 mM KCl/4 mM Tris-ATP/3 mM $MgSO_4$/100 μM sodium molybdate/1 mM NaN_3. The Mg^{2+}-ATPase activity and the vanadate inhibition were not modified by the addition of 10 mM NH_4^+ or 2 μM gramicidin, which dissipate the pH gradient. In the standard medium there are very few free Mg^{2+} ions. When 6 mM free Mg^{2+} was used, the medium was the same except that it contained 3 mM Tris-ATP and 9 mM $MgSO_4$. Additions and modifications to the Mg^{2+}-ATPase reaction mixture, including various concentrations of vanadate and salts, are given in the figures and legends. Stock solutions of vanadate (1 mM) were routinely prepared from sodium orthovanadate (Na_3VO_4). The same results were obtained from sodium metavanadate ($NaVO_3$). The two solutions contained the same vanadate species after equilibration [7]. The reaction was started by addition of 5–10 μg membrane protein and was allowed to proceed for 20 min at 38°C. The liberated P_i was assayed by stopping the reaction with 1 ml Ames reagent [16] containing 0.75% sodium dodecyl sulfate. The control was run without $MgSO_4$ and subtracted from the assay in the presence of $MgSO_4$ so as to calculate the Mg^{2+}-ATPase activity [17]. Whether or not the surface potential of the membrane was depolarized by free Mg^{2+}, the Mg^{2+}-ATPase activity remained 95–100% sensitive to vanadate. Only the inhibition constant was affected by these treatments (see below). Triplicate samples were run for each assay. Protein concentration was estimated by the method of Schaffner and Weissman [18].

Microelectrophoretic measurements

The electrophoretic mobility of single vesicles was measured at 38°C using a Rank Brothers Mark II apparatus fitted out with an ultra-thin-walled cylindrical glass cell. The ultramicroscope illumination allows the detection of small plasmalemma vesicles (approx. 0.3 μm diameter). The medium contained 10 mM Tris, adjusted to pH 6.5 with Mes (Tris is a univalent cation at this pH)/4 mM Tris-ATP/3 mM $MgSO_4$/0.1 mM sodium molybdate and salt at indicated concentrations. The Helmholtz-Smoluchowski relation [19] was used to calculate the zeta potential from the mean microelectrophoretic mobility of 50 vesicles.

Results and Analysis

Mechanisms of inhibition of the Mg^{2+}-ATPase by vanadate and determination of the inhibition constant

The Mg^{2+}-ATPase activity (V) was assayed in the standard incubation medium as a function of the concentration of the substrate Mg^{2+}-ATP (S). The Eadie-Scatchard plots of the data show that the presence of vanadate caused a change in both K_M and V_{max} (Fig. 1A). Furthermore, as shown by the common intercept of the regression lines on the Y-axis, the ratio V_{max}/K_M

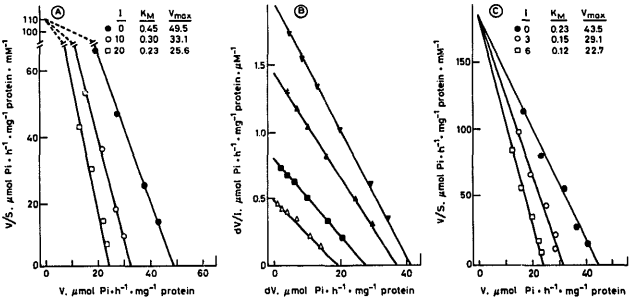

Fig. 1. Vanadate inhibition of the Mg^{2+}-ATPase activity. The assay medium contained 25 mM Tris-Mes (pH 6.5)/25 mM KCl/25 mM KNO_3/0.1 mM sodium molybdate/1 mM NaN_3 and various equimolar concentrations of the substrate (S) Tris-ATP plus $MgSO_4$ and various vanadate concentrations (I). Incubation was for 20 min at 38°C. (A) Eadie-Scatchard of the Mg^{2+}-ATPase activity (V) as a function of S in the absence or the presence of vanadate. (B) Eadie-Scatchard plot of the decrease (dV) of the Mg^{2+}-ATPase activity as a function of vanadate concentration, at different S (mM): 0.3 (△), 0.6 (■) 1.5 (▲) and 3.0 mM (▼). (C) As in (A) but in the presence of 6 mM free Mg^{2+}. Lines were fitted by regression analysis for determining the kinetic and inhibition parameters. K_M, V_{max} and I were expressed in mM, μmol P_i/min per mg protein and μM, respectively. The reciprocal of the slopes in B are indicated in Table I.

remained constant. These results indicate that vanadate inhibition of the plasma membrane Mg^{2+}-ATPase is purely uncompetitive [20]. The inhibition constant (K_I) was graphically determined from the decrease of the Mg^{2+}-ATPase activity (dV) as a function of vanadate concentration (I). When dV/I was plotted as a function of dV, the points lay on a straight line with different slope for each S value (Fig. 1B). The extrapolated values of dV at infinite I concentration were 95–100% of V in the absence of vanadate. These results indicate that the Mg^{2+}-dependent ATP hydrolysis is due totally to the vanadate-sensitive plasmalemma Mg^{2+}-ATPase. For uncompetitive inhibition, V as a function of I is given by [20]

$$V = V_{max} \cdot S/(K_M + S + I \cdot S/K_I) \quad (1)$$

The value of dV as a function of I is then given by

$$dV = A \cdot I/(B + I) \quad (2)$$

where $A = V_{max} \cdot S/(K_m + S)$, and $B = K_I \cdot (1 + K_M/S)$.

The X-axis intercept and the slope of the curves in Fig. 1B thus give the value of A and B, respectively. K_I is the reciprocal of the slope $1/B$, provided that S is saturating. For lower S values, K_I must be calculated by taking into account the value of K_M (Table I). The mean value of K_I was 18.4 ± 2.8 μM (confidence limit for $P = 0.05$). When the medium contained 6 mM free Mg^{2+}, the V_{max} of the Mg^{2+}-ATPase remained nearly unchanged (Fig. 1C), while the K_M decreased from 0.45 to 0.23 mM. Nevertheless, the vanadate inhibition remained uncompetitive (Fig. 1C) with a mean K_I value 8.0 ± 1.8 μM (from Table I). In the following experiments, K_I was determined from vanadate titration at saturating S concentration.

Effect of K^+ and Mg^{2+} on the inhibition constant and determination of the inhibitory form of vanadate

The highest K_I value was obtained in the control medium containing 10 mM Tris-Mes/4 mM Bistris propane-ATP/0.1 mM sodium molybdate/3 mM Mg^{2+} (Table II). The addition of 10 mM KCl caused a 3-fold decrease of K_I. Only a small additional decrease of K_I

TABLE I

Determination of the vanadate inhibition constant of the steady-state activity of the Mg^{2+}-ATPase from Eadie-Scatchard plots

The control assay medium is described in the legend to Fig. 1. To obtain 6 mM free Mg^{2+}, 6 mM $MgSO_4$ was added to equimolar concentrations of Tris-ATP plus $MgSO_4$ as indicated. For each ATP concentration, S, the reciprocal of the slope of the Eadie-Scatchard plot, B, was determined from vanadate titration. The inhibition constants, K_I, were calculated from the equation $K_I = B/(1 + K_M/S)$ with $K_M = 0.44$ mM for the control and 0.23 mM for the control in the presence of 6 mM free Mg^{2+}. All values are μM.

S (mM)	Control		6 mM free Mg^{2+} added	
	B	K_I	B	K_I
0.3	40.0	16.2	16.7	9.4
0.6	34.5	19.9	11.2	8.1
1.5	25.6	19.8	7.7	6.7
3.0	16.1	14.6	8.5	7.9

TABLE II

Effect of ions on apparent and intrinsic inhibition constant for vanadate and zeta potential

The control assay medium contained 10 mM Tris-Mes (pH 6.5)/4 mM Tris-ATP/3 mM $MgSO_4$/0.1 mM Na molybdate and various salts as indicated. For the 6 mM free Mg^{2+} condition, 9 mM $MgSO_4$ and 3 mM Tris-ATP were used. For each condition, the apparent inhibition constant was determined from vanadate titration. The zeta potential was determined from microelectrophoretic mobility of membranes. Calculated intrinsic inhibition constant, K_{Ii} was obtained using the Boltzmann law (see Eqn. 3), assuming different valencies, z for the inhibiting form of vanadate.

Ion added (mM)	Zeta potential (mV)	Observed K_I (μM)	Observed K_{Ii} [a] (μM)	Calculated K_{Ii} [b] (μM)		
				$z=-1$	$z=-2$	$z=-3$
None	−18.7	98.6	22.6	49.1	24.4	12.1
10 Na^+	−15.1	77.2	n.d.	44.0	25.0	14.2
10 K^+	−15.8	34.5	10.1	19.1	10.6	5.9
10 K^+ [c]	−11.2	23.3	n.d.	15.3	10.1	6.7
50 K^+	−10.8	22.8	10.5	15.2	10.2	6.8

[a] In media supplemented with 6 mM free Mg^{2+} in order to annihilate electrostatic interactions in the vicinity of the Mg-ATPase.
[b] Calculated from Eqn. 3 with the valencies, z, corresponding to the different vanadate species.
[c] In the presence of 50 mM Tris-Mes.

was obtained at higher K^+ concentration (50 mM), or when the medium containing 10 mM K^+ was supplemented by 50 mM Tris-Mes. On the other hand, the addition of 10 mM NaCl induced only a 1.3-fold decrease of K_I. The highest absolute value of zeta potential was also obtained in the control medium. The addition of 10 mM KCl (or NaCl) and 50 mM KCl (or Tris-Mes) caused respectively a 1.2 and 1.7-fold decrease of the zeta potential (Table II). Assuming that the accessibility of the various anionic forms of vanadate to the binding site depends on the zeta potential (ζ) obtained from microelectrophoretic measurements, the intrinsic inhibition constant (K_{Ii}, i.e., the inhibition constant in the absence of electrostatic interactions) may be calculated from the Boltzmann law [12].

$$K_{Ii} = K_I \cdot \exp(-z\psi/kT) \qquad (3)$$

where z, k and T are, respectively, the valency of the vanadate species, the Boltzmann constant and the absolute temperature. Of course, three sets of K_{Ii} values were obtained, depending on the value chosen for z. Whatever the valency attributed to the active form, the value of K_{Ii} decreased approx. 2-fold upon addition of 10 mM K^+ (in the presence of 10 or 50 mM $Tris^+$) or 50 mM K^+, while K_{Ii} was not modified upon addition of 10 mM NaCl (Table II). This suggests either that the zeta potential obtained from microelectrophoretic mobility underestimated the electrostatic effect of K^+ on the vanadate binding site of the Mg^{2+}-ATPase or that K^+ had a direct (conformational ?) effect on the enzyme, which modified K_{Ii}.

The intrinsic inhibition constant was estimated more directly, using the properties of free Mg^{2+}. Indeed, we have previously shown [12] that 6 mM free Mg^{2+} totally annihilated the surface charge of the proteins of corn root plasma membrane and suppressed the electrostatic repulsion of Mg-ATP in the vicinity of the Mg^{2+}-ATPase. This depolarization comes from both the binding of Mg^{2+} on proteins (but not on lipids) and the electrostatic screening effect of the bivalent cation. In this condition, the intrinsic Michaelis constant for Mg^{2+}-ATP could be measured directly. In a similar way, K_{Ii} could be directly obtained in the presence of 6 mM free Mg^{2+}. Under this condition, a 2-fold decrease of K_{Ii} was observed upon K^+ addition (Table II). Furthermore, the values of K_{Ii} obtained in the absence

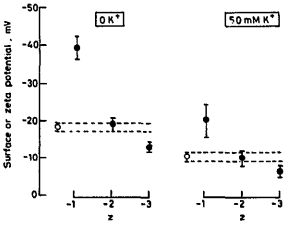

Fig. 2. Comparison of the zeta potential and the surface potential calculated from vanadate inhibition constant. The determination of the zeta potential is described in Materials and Methods. Eqn. 3 was used for calculating the surface potential from the vanadate inhibition constants in Table II, assuming different valencies, z, for the inhibiting form of vanadate. Medium contained 10 mM Tris-Mes (pH 6.5)/4 mM Tris-ATP/3 mM $MgSO_4$/0.1 mM sodium molybdate with or without 50 mM KCl for determining microelectrophoretic mobility and the apparent vanadate inhibition constant. The intrinsic inhibition constant was determined in the same medium, except that the concentrations of Tris-ATP and $MgSO_4$ were 3 and 9 mM, respectively. The confidence limits (means ± S.D.) of K_I and K_{Ii} were used to obtain the higher and lower estimates of calculated ψ. For the zeta potential, the bars are the confidence limits for $p = 0.05$. (○) zeta potential; (●) surface potential.

or in the presence of K^+ (23 μM and 10 μM, respectively) were in good agreement with those previously calculated, assuming that the inhibitory species of vanadate is a bivalent anion.

The above set of experimental results on K_I can be used together with K_{II} determined in the presence of free Mg^{2+}, following a different analysis. Indeed, the surface potential can be calculated from the couples (K_I, K_{II}) obtained in the presence or in the absence of K^+, assuming different z values. Thereafter, it can be compared to the zeta potential from microelectrophoretic experiments. Again, the potentials are in good agreement, assuming the active form of vanadate is a bivalent anion (Fig. 2).

Discussion

The monophasic curves of Fig. 1 support the hypothesis of a single Mg^{2+}-ATPase on the corn root plasma membrane, inhibited by vanadate. Similar conclusions have been obtained from crude plasma membranes in 80000 × g pellet [21], or after Triton X-100 treatment of phase-partitioned plasma membranes according to Galtier et al. [22] (data not shown). The results of Fig. 1 indicate that Mg^{2+}-ATPase of corn root plasma membranes is uncompetitively inhibited by vanadate. The slopes of the plots of Fig. 1B give the reciprocal values of the apparent inhibition constant K_I for uncompetitive inhibition, provided the substrate concentration is saturating (parameter B in Eqn. 2).

For the same material, noncompetitive inhibition and a high K_I value (233 μM in the presence of 50 mM K^+) have been reported [23]. It has been shown that the yeast plasma Mg^{2+}-ATPase displays noncompetitive inhibition at low free Mg^+ concentration and a mixed uncompetitive-noncompetitive inhibition at high concentration [24]. Nevertheless. the results of Fig. 1C indicate that the Mg^{2+}-ATPase remained uncompetitively inhibited in the presence of 6 mM free Mg^{2+}. The precise origin of the above discrepancy remains unclear, as already reported elsewhere [4]. It is to be pointed out that a variety of types of inhibition have been reported for plant and animal Mg^{2+}-ATPases. Noncompetitive [23,25] and uncompetitive [4] inhibitions were found in the case of the Mg^{2+}-ATPase of plant plasma membranes. Competitive inhibition has been reported for gastric H^+/K^+-ATPase [10] and Na^+/K^+-ATPase [2]. Furthermore, large variations of the vanadate inhibition constant value have been also reported, from 0.01 to 10 μM [2,8,10] for animal Mg^{2+}-ATPase and from approx. 10 to 200 μM [4,23] for plant Mg^{2+}-ATPase.

The addition of Mg^{2+} or K^+ caused a decrease in the apparent inhibition constant (Table II). The effect of the two ions was additive and their simultaneous addition caused a 10-fold decrease in K_I, suggesting distinct interactions at the vanadate binding site. Indeed, the electrostatic analysis suggests that free Mg^{2+} only increases the accessibility of the vanadate anion to the catalytic site by annihilating the repulsion due to the negative surface charge, without modifying K_{II}. On the other hand, the K_{II} value decreases 2-fold in the presence of K^+ (and not in the presence of Na^+) due to direct (i.e., non-electrostatic) effect on the Mg^{2+}-ATPase. Finally, this electrostatic analysis suggests that the vanadate-inhibiting form of the plasmalemma Mg^{2+}-ATPase is a bivalent anion.

The above conclusions on the effects of ions on the plasmalemma Mg^{2+}-ATPase inhibition clearly depend on the validity of two main assumptions. The first assumption is that the intrinsic inhibition constant is directly measured in the presence of 6 mM free Mg^{2+} (i.e., the surface potential in the vicinity of the Mg^{2+}-ATPase may be taken as zero in this condition). We have previously shown [12] that the microelectrophoretic mobility of latex beads coated with the proteins extracted from plasmalemma was reduced to zero by adding 6 mM free Mg^{2+}. The accessibility of the anion Mg^{2+}-ATP to the catalytic site on native membrane was improved by the screening effect of salts in the absence of free Mg^{2+}, but not in its presence. Furthermore, the intrinsic Michaelis constant measured in the presence of free Mg^{2+} was the same as the one calculated from the zeta potential in its absence. Similarly, the vanadate inhibition constant became insensitive to the ionic strength when the medium contained 6 mM free Mg^{2+} (Table II). This observed intrinsic inhibition constant agrees with the calculated one only if a bivalent anion is assumed to be the inhibiting species. The similarity of the present effects of free Mg^{2+} on the vanadate–enzyme interaction with the previously reported effects on the Mg^{2+}-ATP–enzyme interaction [12] is to be noted, since vanadate and Mg^{2+}-ATP are known to bind on the same site [2,8].

The second assumption is that the zeta potential given by microelectrophoresis is adequate for the analysis of the electrostatic interactions in the vicinity of the Mg^{2+}-ATPase. The same surface-charge density of corn root plasma membranes in the absence of bivalent cations and at pH 6.5 has been previously obtained by three independent methods (binding of lipophilic anilinonaphthalenesulfonate probe, microelectrophoretic mobility of vesicles and affinity of protein for substrate) [12]. From this result, we have concluded that the electrostatic potential was uniform over the membrane surface, as already reported for horse bean membranes [13] and various animal membranes [26,27]. The zeta potential given by microelectrophoresis thus appears valuable for an analysis of electrostatic interactions on the vanadate and the Mg^{2+}-ATPase in the absence of bivalent cation and at pH 6.5. Finally, the first assumption (K_{II} can be measured directly in the presence of 6 mM free Mg^{2+}) allows the calculation of

the surface potential from K_I and K_{Ii} values and Eqn. 3 and its comparison with the measured zeta potential. The second assumption (uniformity of the surface charge) can then be fulfilled only if the inhibiting vanadate species is assumed to be a bivalent anion (Fig. 2).

Concerning the direct effect of K^+ on K_{Ii}, a general feature of the Mg^{2+}-ATPase of plant plasma membrane is its stimulation by K^+ [28]. For instance, corn root plasmalemma Mg^{2+}-ATPase is 60% stimulated by K^+ [12]. Furthermore, an absolute K^+ requirement has been recently reported for catalytic function of highly purified Mg^{2+}-ATPase from the same material [29]. The direct effect of K^+ on the plant plasmalemma Mg^{2+}-ATPase has been recently reviewed by Briskin [11]. Two forms of phosphorylated intermediate (i.e., an E1 form which is sensitive to ADP and an E2 which is not) have been demonstrated, and K^+ stimulates the rate of the ADP-insensitive phosphoenzyme breakdown. Thus, the direct effect of K^+ on the intrinsic vanadate inhibition constant (Table II) seems likely to be related to conformational change of the enzyme.

A variety of vanadate species have been shown to be capable of inhibiting different enzymes. Various phosphotransferases [6,30,31] are inhibited only by the octahedrally coordinated decameric vanadium polyanion (present only when the total vanadate concentration is in the millimolar range). All the tetrahedrally coordinated oligomeric forms inhibit phosphorylases, while monomeric species are slightly activating [32]. The present analysis suggests that the inhibiting vanadate species is a bivalent anion. At neutral pH, the dimeric and trimeric vanadates, $H_4V_2O_7$ and $H_3V_3O_9$, do not exist in the bivalent ionization state but in the trivalent state. Furthermore, the very low inhibition constants of animal Mg^{2+}-ATPases (0.01–10 μM) strongly argue for monomeric vanadate as the inhibitory species, since oligomeric forms of vanadate come from polymerization reactions at higher vanadate concentration. When the total vanadate concentration is in the range 10–100 μM at pH 7, both monovalent ($H_2VO_4^{1-}$) and bivalent (HVO_4^{2-}) ionization forms of orthovanadic acid are present, but the former is prevalent (pK_a is about 8) [33]. At pH higher than 8, the bivalent form becomes predominant. Unfortunately, most of the published work has been performed at pH values where the two ionization states co-exist and thus does not indicate the inhibitory species. A study of the myosin Mg^{2+}-ATPase, performed at pH 8.5, shows that this enzyme can be inhibited by HVO_4^{2-} [34]. Such an experiment cannot be carried out with corn root plasmalemma Mg^{2+}-ATPase because its maximum activity is observed at pH 6.5. The K_{Ii} values given in Table II have been obtained from the total concentration of the various vanadate species in the solution and not from the HVO_4^{2-} concentration. Since the pK_a of the dissociation of $H_2VO_4^{1-}$ is about 8.0, HVO_4^{2-} represents only about 3% of the total vanadate concentration. Thus, the actual K_{Ii} values should be probably about 30-fold lower than those given in Table II.

In conclusion, the vanadate inhibitor constant for uncompetitive inhibition of the steady-state activity of the corn root plasmalemma Mg^{2+}-ATPase is an apparent constant, which depends on electrostatic interactions. The large decrease of the inhibition constant in the presence of free Mg^{2+} seems due entirely to indirect depolarizing effects of this cation (screening and binding effects). This has allowed us to determine the intrinsic inhibition constant. On the other hand, a direct and specific effect of K^+ on the enzyme causes a 2-fold decrease of the intrinsic inhibition constant. Finally, the analysis of two kinds of data, namely determination of the inhibition constant and of the surface potential, indicates that the Mg^{2+}-ATPase of corn root plasmalemma is inhibited by the monomeric bivalent anion HVO_4^{2-}.

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