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Some properties of the H-ATPase activity present in root plasmalemma of *Avena sativa* L. Two different enzymes or one enzyme with two ATP sites?

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The effects of Mg^{2+}, K^+ and ATP on a H-ATPase activity from a native plasmalemma fraction of oat roots were explored at 20°C and pH 6.5. In the presence of 3 mM ATP and no K^+, H-ATPase activity vs. $[Mg^{2+}]$ approached a monotonic activation but it became biphasic, with a decline above 3 mM Mg^{2+}, in the presence of 20 mM K^+. Mg^{2+} inhibition occurred also in K-free solutions when [ATP] was lowered to 0.05 mM. Also, an apparent monotonic H-ATPase activation by $[K^+]$ at 3.0 mM ATP was transformed in biphasic (inhibition by high $[K^+]$) when [ATP] was reduced to 0.05 mM. The best fits of the ATP stimulation curves of hydrolysis satisfied the sum of two Michaelian functions where that with higher affinity had lower V_{mx}. Taking into consideration all conditions of activity assay, the high-affinity component (1) had a K_m about 11–16 μM and a V_{mx} around 0.14–0.28 μmol P_i/mg per min whereas that with lower affinity (2) had a K_m of 220–540 μM and a V_{mx} of 0.5–1.0 μmol P_i/mg per min. K_{m2} was markedly affected by the $[K^+]$ and $[Mg^{2+}]$; at optimal concentrations of these cations (1 mM Mg^{2+} and 10 mM K^+) it had a value of 235 ± 24 μM which was increased to 540 ± 35 μM at 20 mM $[Mg^{2+}]$ and 60 mM $[K^+]$. In addition, V_{mx1} was reduced to about a half when the concentrations of Mg^{2+} and K^+ were increased to inhibitory levels. These results could be explained by the existence of two different enzymes or one enzyme with two ATP sites. In the second case, we could not tell at this stage if both are catalytic or one is regulatory.

Introduction

The H-ATPase from higher plants is a transport enzyme likely to belong to the E_1-E_2 group; its function is to actively extrude protons from the cytosol [1]. The bulk of information on H-ATPases has been obtained in yeast and fungi, and only recently there has been some work dealing with that of higher plants [2–4]. The presence of Mg^{2+} is absolutely required for its activity, and the optimal $MgCl_2$ concentration coincides with that of ATP. There is no agreement about the true substrate, and both MgATP complex [5,6] and free ATP [7] have been suggested; the second alternative leaves free Mg^{2+} as an essential activator. Another Mg^{2+} site, where this cation acts as inhibitor has been proposed [8]. At non-limiting $MgCl_2$ concentrations, the half-maximal activity is obtained with about 0.3 mM ATP [3]. Most of the available evidence indicates the existence of a single, low affinity, ATP site in the H-ATPase [1,9,10]. There are only two reports we are aware of where two ATP binding sites are considered: one of them suggests positive cooperativity between them with a $K_{1/2}$ ranging from 0.6 mM to 2.0 mM [11]; the other proposes two catalytic loci with K_m values of 0.56 mM and 4.9 mM [6]. Consequently, the high-affinity catalytic site present in all other E_1-E_2 transport ATPases [12] seems to be missing in this enzyme.

Although a large fraction of H-ATPase activity does not require other ligands besides Mg^{2+} and the substrate, the presence of K^+ increases the rate of ATP hydrolysis. This potassium stimulation might involve an increase in the rate of dephosphorylation [2] or a stimulation of the E_1P-E_2P transition [13]. An ADP-stimulated dephosphorylation has also been reported, and the ADP-sensitive phosphoenzyme has been tentatively identified with an E_1-P conformation [13,14].

The experiments described in this work were per-

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formed in order to study in detail the effects of Mg^{2+}, K^+ and ATP on the activity of native H-ATPase from higher plants. They show interactions between the three aforementioned ligands and the enzyme, while the resulting effect (stimulation, inhibition) depends on their relative concentrations. In addition, the complex ATP activation curves could imply the existence of (i) two isoforms of the enzyme, or (ii) a single species with two ATP sites.

Methods

Roots from *Avena sativa* L., grown for 7 days at 20 ± 2 °C in the dark, were cut and homogenized as in Ref. 3. After centrifugation for 20 min at $5000 \times g$, the pellet was discarded and the supernatant was centrifuged for 30 min at $85000 \times g$. The second pellet was resuspended (protein concentration about 7 mg/ml) in 20% glycerol/1 mM EDTA/1 mM dithiothreitol/10 mM Tris-HCl (pH 7.5 at room temperature) and centrifuged again on a sucrose gradient (33–46%) for 120 min at $85000 \times g$. The microsomal fraction obtained from the interface was washed once according to Ref. 15. The resulting membrane fraction was stored in the glycerol buffer medium at −20 °C; under these conditions the H-ATPase activity (about 1 μmol P_i/mg per min at 20 °C) remained stable for 3–4 months.

Protein content was measured with the method of Bradford [16] using BSA as standard. The ATPase activity was assayed at 20 °C. The amounts of ATP hydrolyzed were estimated on the basis of the radioactivity released from [γ-^{32}P]ATP of known specific activity [17]. The incubation solutions contained 50 mM Tris-Mes (pH 6.5 at 20 °C)/0.1 mM Tris-EGTA and 1 mM dithiothreitol; the concentrations of the other ligands ($MgCl_2$, KCl and ATP) depended on the experiment designs and are indicated in the corresponding figure legends. Routinely, the activity of acid phosphatases and mitochondrial ATPase were prevented by the addition of 0.1 mM ammonium molybdate and 5 mM sodium azide, respectively. In some cases (ATP activation curves) a possible contamination with vacuolar H-ATPase was checked by including 20 mM nitrate as a K^+ salt. The fractional ATPase inhibition due to nitrate averaged 15% and was independent of the ATP and enzyme concentrations as well as the incubation time. This inhibition is much smaller than that expected for tonoplast ATPase [18,19]; actually, it is of the order observed for H-ATPase even in highly purified plasma membrane preparations (see above references). Therefore, although some tonoplast enzyme might be present in our preparation, it is extremely unlikely that it would affect the results.

Immunological cross-reactivity of the plant membrane preparation with pig kidney Na,K-ATPase was assayed using a rabbit antisera prepared in our laboratory in a 1 : 200 dilution. The general procedure was similar to that described by Towbin et al. [20] as modified by Burnette [21] using 125-iodine labelled protein A as a marker.

Phosphorylation from [γ-^{32}P]ATP was carried out for 20 s at 0 °C adding 250 μg total protein into 0.25 ml of a solution containing 50 mM Tris-Mes (pH 6.5 a 0 °C), 1 mM $MgCl_2$, 0.2 mM ATP with and without 20 mM KCl. The reaction was stopped with 0.5 ml of an ice-cold media containing 12% (w/v) perchloric acid/10 mM inorganic phosphate/5 mM unlabelled ATP [22]; the tubes spent 15 min in an ice-cold bath before any further treatment. Dephosphorylation was also followed at 0 °C and phosphoenzyme formation was stopped adding CDTA (enough to chelate all free Mg^{2+}) plus the ligand which effect was to be explroed; the reaction was stopped 3 s thereafter. The separation of the phosphoproteins present in the membrane fraction was attained by lithium dodecyl sulphate polyacrylamide (5%) gel electrophoresis [23]. The denatured samples were centrifuged in a cold room for 15 min at $12000 \times g$ in an eppendorf microcentrifuge. The pellets were washed three times with the stopping solution, resuspended in the appropriate buffer (pH 2.4) and applied on top of the gels. The electrophoretic runs lasted 6 h at 25 mA and 4 °C. Each lane was sliced in 3 mm segments which were counted in a liquid scintillation counter using toluene based scintillator. The areas under the phosphorylation peaks were measured with a graphic tablet attached to an Apple II computer. Partially purified pig kidney Na,K-ATPase, subjected to the same treatment, was used as a radioactive marker for a phosphorylated α subunit. Additional molecular weight markers (stained with Coomassie blue) were β-galactosidase (116000), phosphorylase *a* (94000), bovine serum albumin (66000) and albumin egg (45000).

All solutions were prepared with deionized bidistilled water and reagent grade chemicals. Carrier free [^{32}P]P_i was purchased from the Comisión Nacional de Energía Atómica of Argentina. [γ-^{32}P]ATP was labelled according to the method of Glynn and Chappell [24]. The concentration of ionized Mg^{2+} was calculated on the basis of the Arsenazo III spectra shift measured in the same media used in the experiments [25]. Actually, in the standard ATPase assay solutions (50–70 mM ionic strength, pH 6.5, 20 °C) the Mg^{2+} dye dissociation constant was 4.1 ± 0.5 mM and 6.3 ± 0.7 mM in the absence and presence of 20 mM KCl, respectively, whereas the Mg · ATP dissociation constant was 0.6 ± 0.1 mM in both instances.

Duplicate, and some times triplicate, samples were run in each case, and all experiments were repeated at least once. Curve fitting was performed with a non-linear regression computing program using the least-squares criterion (Scopfit, National Biomedical Simula-

tion Resource, Duke University Medical Center, U.S.A.).

Results

General characterization of the membrane fragments

The plasma membrane fraction obtained from roots of *Avena sativa* L. showed an ATPase activity with the characteristics described for the H-ATPase from plants [1,3,26]. In the presence of 5 mM $MgCl_2$ and 3 mM ATP the maximal rate of hydrolysis was observed at pH 6.5. At the optimal pH and 20 °C, the activity was 30–50% stimulated by 20 mM K^+. On the other hand, this ATPase was sensitive to the inhibitors that act upon the H-ATPases from plants: it was about 90% inhibited by 100 μM vanadate (see Refs. 4 and 12) and 100 μM diethylstilbestrol and totally blocked by 50 μM erythrosine (see Ref. 1). It is important to point out the existence in this membrane fraction of a protein of molecular weight around 100000 which shows cross reactivity with a rabbit polyclonal antibody against the catalytic subunit of Na,K-ATPase from pig kidney (Fig. 1). This, and the results reported below, suggest that the molecule responsible for the effects described here has structural homology with an animal transport ATPase of the E_1-E_2 type [10].

Fig. 2 shows a LDS-PAGE run of the membrane fragments phosphorylated from ATP in the presence of

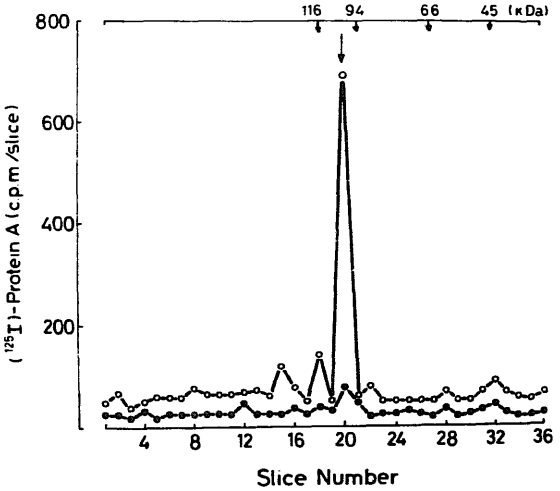

Fig. 1. Cross-reactivity of rabbit antisera against Na,K-ATPase from pig kidney (open circle) with an M_r = 100000 plasma membrane fraction from oat roots. Aliquots of 75 μg total protein were separated by PAGE using 7.5% (w/v) polyacrylamide running gel and electroblotted onto nitrocellulose. The immunological detection was performed on the basis of 125-iodine labelled protein A. The arrow indicates the migration distance corresponding to the α subunit of the Na,K-ATPase. Filled circles represent duplicate samples of oat membranes reacting with control rabbit sera. Molecular weight markers (stained with Coomassie blue) were β-galactosidase (116000), phosphorylase *a* (94000), bovine serum albumin (66000) and albumin egg (45000). Each point corresponds to a 2.5 mm slice. See Methods for details.

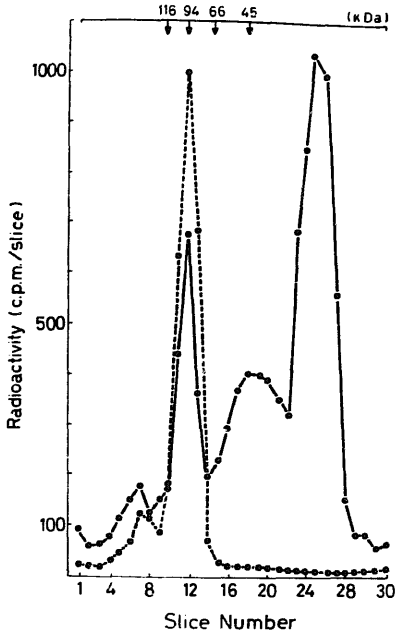

Fig. 2. LDS-PAGE acid stable radioactivity profile of a plasma membrane preparation from oat roots exposed to [γ-^{32}P]ATP. The solid line corresponds to the oat roots membranes incubated for 20 s at 0 °C and pH 6.5 in the presence of 1 mM $MgCl_2$, 50 mM Tris-Mes, 0.5 mM EGTA and 200 μM [^{32}P]ATP. A sample of partially purified pig kidney Na,K-ATPase phosphorylated from [^{32}P]ATP in the presence of 120 mM NaCl and 1 mM $MgCl_2$ (broken line) has been included for comparison. The polyacrylamide concentration (w/v) was 5%. Molecular weight markers (stained with Coomassie blue) were β-galactosidase (116000), phosphorylase *a* (94000), bovine serum albumin (66000) and albumin egg (45000). Each point corresponds to a 3.0 mm slice. See Methods for details.

$MgCl_2$ under steady-state conditions (20 s at 0 °C). Three major acid stable components can be observed, corresponding to M_r 100000, M_r 44000 and M_r 23000; the latter value is a rough approximation for the peak migration is close to that of the tracking dye. The migration distance of the lower mobility peak coincides exactly with that of the E-P intermediate obtained with pig kidney Na,K-ATPase; in addition, likewise the pig phosphoenzyme, it disappeared completely upon treatment with hydroxylamine (not shown), suggesting the existence of an acyl phosphate bond, something that has been described for H-ATPases from plants and fungi [5,27]. Furthermore, this component was decreased to a half when phosphorylation was performed in the presence of 20 mM KCl and it was totally abolished by 100 μM vanadate, with and without KCl (not shown).

The breakdown of the M_r 100000 phosphoprotein was explored by determining the relative changes in the areas under the peaks 3 s after phosphorylation has been halted with CDTA. The results showed the following: (i) In the absence of any added ligand the spontaneous breakdown amounted to about 50%; this corresponds to a pseudo rate constant of around 0.23 s^{-1}. (ii) Interestingly enough, and within the limitations of the method, this phosphoprotein seems similarly sensitive

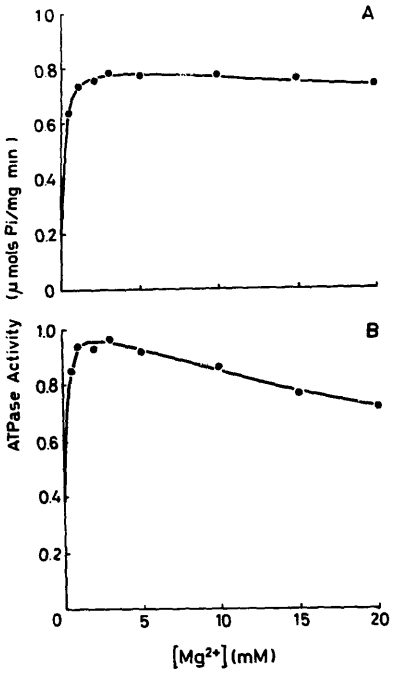

Fig. 3. Effects of ionized Mg^{2+} concentrations on H-ATPase activity of a plasma membrane preparation from oat roots incubated in the presence of 3.0 mM ATP without (A) or with (B) 20 mM KCl. Temperature was 20°C and pH 6.5. Each point is the mean of duplicate samples corresponding in each case to conditions A and B tested in parallel experiments (same symbols). The lines through the points were drawn by eye.

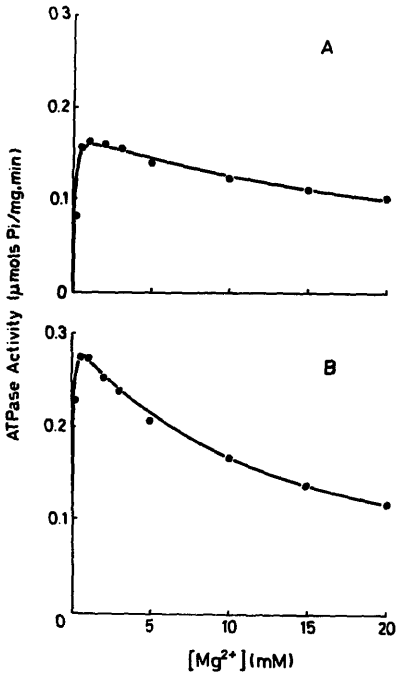

Fig. 4. Experiment similar to that illustrated in Fig. 3 but with an ATP concentration of 0.05 mM. Each point is the mean of duplicate samples corresponding in each case to conditions A and B tested in parallel experiments (same symbols). The lines through the points were drawn by eye. Note that the symbols close to the vertical axis represent 0.05 mM Mg^{2+}.

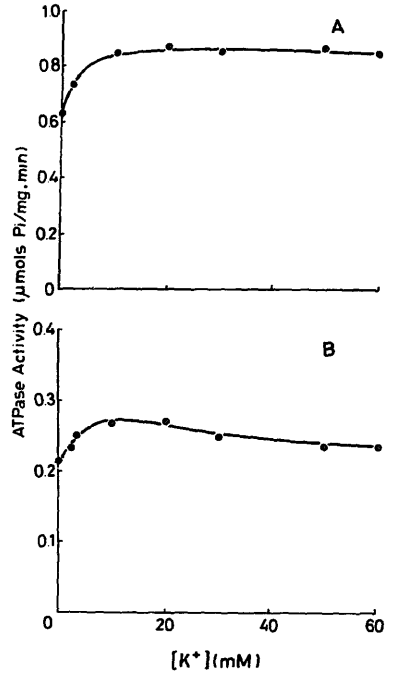

Fig. 5. Effects of K^+ concentrations at constant 5 mM ionized Mg^{2+} on H-ATPase activity of a plasma membrane preparation from oat roots incubated in the presence of 3.0 mM (A) or 0.05 mM (B) ATP. Temperature was 20°C and pH 6.5. Each point is the mean of duplicate samples corresponding in each case to conditions A and B tested in parallel experiment (same symbols). The lines through the points were drawn by eye. Note that the symbols on the vertical axis represent K-free conditions.

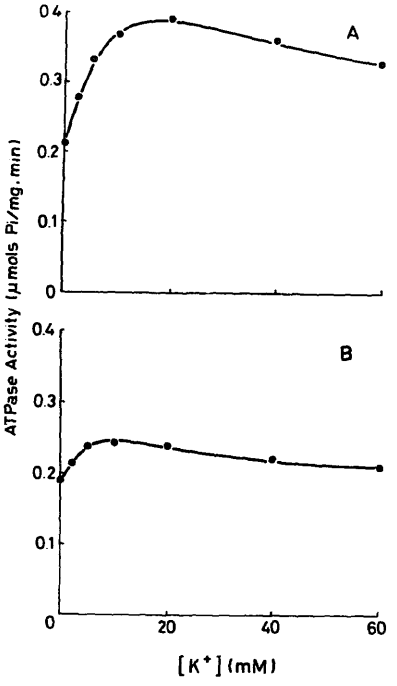

Fig. 6. Effects of K^+ concentrations on H-ATPase activity of a plasma membrane preparation from oat roots incubated with 0.05 mM ATP in the presence of 1 mM (A) or 20 mM (B) ionized Mg^{2+}. Temperature was 20°C and pH 6.5. Each point is the mean of duplicate samples corresponding in each case to conditions A and B tested in parallel experiment (same symbols). The lines through the points were drawn by eye. Note that the symbols on the vertical axis represent K-free conditions.

to both K^+ and ADP. Thus, the residual phosphorylation after 3 s averaged 16% in the presence of 20 mM KCl and 10% when 1 mM ADP was added (pseudo rate constants of 0.61 s^{-1} and 0.77 s^{-1}, respectively). On the other hand, the simultaneous presence of KCl and ADP did not increase the breakdown rate observed with either ligand alone.

The remaining phosphorylation peaks were insensitive to hydroxylamine, their dephosphorylation rates were much slower than that of the M_r 100000 and not affected by either KCl or ADP. The third peak disappeared when the membranes were pretreated with Triton X-100 (2 mg per mg protein for 10 min at 20°C). Finally, when phosphorylation took place under conditions that lead to complete hydrolysis of the [γ-^{32}P]ATP initially present (30 s at 20°C), the M_r 100000 region was absent whereas the other two appeared unaltered (not shown). This is another indication of the faster dephosphorylation rate of the M_r 100000 peak.

Mg^{2+}, K^+ and ATP interactions

The presence of magnesium is an absolute requirement for this H-ATPase activity. On the other hand, the activity observed in the presence of K^+ (at least up to 60 mM KCl) is always larger than that seen in K-free solutions. However, the rate of ATP hydrolysis is a complex function of Mg^{2+}, K^+ and ATP concentrations.

Figs. 3A and B show that the response of H-ATPase activity to ionized Mg^{2+} concentration at 3 mM ATP is difficult to distinguish from a monotonic activation in the absence of K^+, but it becomes biphasic in the presence of 20 mM KCl with a decline in activity above 3 mM Mg^{2+}. In other words, K^+ appears to potentiate an inhibiting action of Mg^{2+} acting on site/s of low apparent affinity. Nevertheless, the presence of K^+ is not an absolute requirement for Mg^{2+} inhibition, for as it is shown in Fig. 4A that inhibition occurs also in K^+-free solutions provided the concentration of ATP is

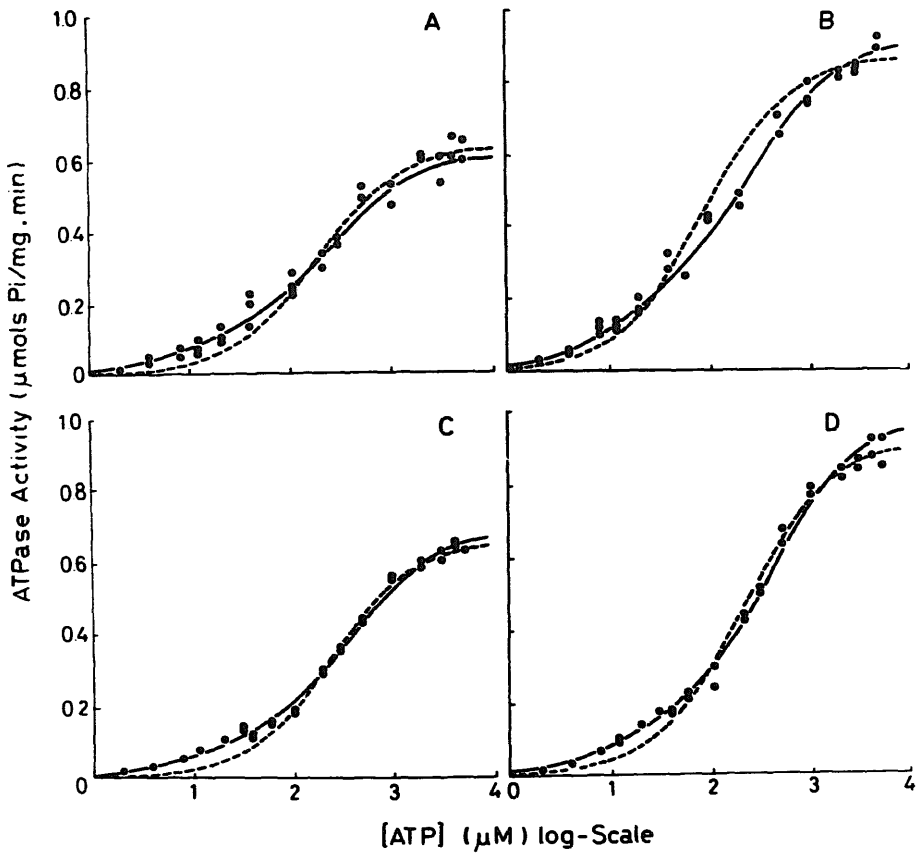

Fig. 7. H-ATPase activity in a native membrane preparation from oat roots as a function of the ATP concentration. The assays were carried out as indicated in Methods in the following conditions: (A) 1 mM ionized Mg^{2+} in the absence of K^+ ions; (B) 1 mM ionized Mg^{2+} and 20 mM KCl; (C) 10 mM ionized Mg^{2+} with no KCl; (D) 10 mM ionized Mg^{2+} and 20 mM KCl. Each point is the mean of duplicate or triplicate samples from different experiments. The lines through the points are the best fit to a single Michaelian (Eqn. 1, broken line) or to the sum of two Michaelians (Eqn. 2, solid line). The values of each fitting parameter for the two sites model were: (A) $V_{mx1} = 0.12$ μmol/mg per min; $K_{m1} = 13$ μM; $V_{mx2} = 0.52$ μmol/mg per min; $K_{m2} = 241$ μM. (B) $V_{mx1} = 0.16$ μmol/mg per min; $K_{m1} = 16$ μM; $V_{mx2} = 0.70$ μmol/mg per min; $K_{m2} = 285$ μM. (C) $V_{mx1} = 0.13$ μmol/mg per min; $K_{m1} = 12$ μM; $V_{mx2} = 0.59$ μmol/mg per min; $K_{m2} = 333$ μM. (D) $V_{mx1} = 0.14$ μmol/mg per min; $K_{m1} = 13$ μM; $V_{mx2} = 0.85$ μmol/mg per min; $K_{m2} = 382$ μM.

lowered (from 3 mM to 0.05 mM in our experiments). In addition, the simultaneous presence of K^+ and low ATP (0.05 mM) increases the inhibiting effect of high [Mg^{2+}] (Fig. 4B).

Conversely, an apparently monotonic dose-response curve of H-ATPase as a function of K^+ concentration at 3.0 mM ATP and 5 mM [Mg^{2+}] (Fig. 5A) can be transformed in biphasic (inhibition developing at high potassium concentrations) when [ATP] is lowered to 0.05 mM; this is observed at 5 mM (Fig. 5B), and 1 mM or 20 mM (Figs. 6A and B) [Mg^{2+}]. Furthermore, the fractional magnitude of the decline observed at 60 mM KCl increases together with the [Mg^{2+}] (see the same figures). Interestingly enough, the maximal fractional stimulation attained with K^+ is higher at 1 mM than at 5 mM or 20 mM [Mg^{2+}].

ATP stimulation curves of H-ATPase activity

Letting aside ATP, the potentiation between K^+ and Mg^{2+} in producing inhibition would not be difficult to explain if both cations enter the enzyme as activators and are released as 'products'. What is not obvious is the mechanism for the increase in their inhibiting power following a reduction in the concentration of ATP. One possibility is competitive interactions, provided there are more than one K^+ and Mg^{2+} [6,8] sites. On the other hand, it cannot be excluded that in the H-ATPase cycle, in the presence and absence of K^+, there is a conformational transition between an enzyme form refractive and a form susceptible to phosphorylation from ATP. In this case, it is not unlikely that that transition, as it happens with other cations ATPases [12,28,29], is accelerated by ATP acting with low affinity (regulatory role). In order to look into that possibility, we explored in detail the dose-response curves of H-ATPase activity versus [ATP] under different magnesium and potassium concentrations. In Fig. 7 the conditions investigated were 1 mM Mg^{2+}, without (A) or with 20 mM KCl (B) and 10 mM Mg^{2+} without (C) or with 20 mM KCl (D). Two models were used to fit the data points. One

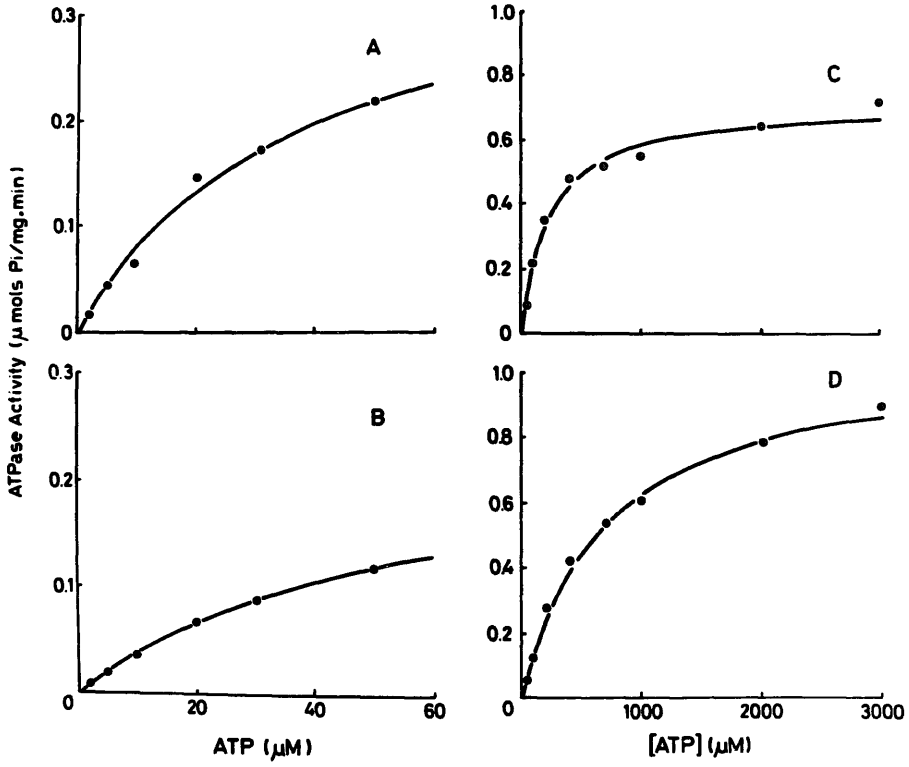

Fig. 8. Dose-response curves for ATP stimulation of the high (left panels) and low (right panels) ATP affinity components of H-ATPase from a native membrane preparation of oat roots. The assays were carried out as indicated in Methods in the following conditions: (A) and (C), 1 mM ionized Mg^{2+} and 10 mM KCl; (B) and (D), 20 mM ionized Mg^{2+} and 60 mM KCl. The points in the figure were estimated as follows: first, all experimental values were used to calculate, by curve fitting, the V_{mx} and K_m of both components; then, to every experimental value, that corresponding to the component not being considered (on the basis of the kinetic parameters and [ATP]) was subtracted. The lines through the points are the best fits to single Michaelian functions. The mean values, plus minus the S.E., for the kinetic parameters obtained from three different experiments were: (A) $V_{mx1} = 0.28 \pm 0.03$ μmol P_i/mg per min, $K_{m1} = 13 \pm 2$ μM; (B) $V_{mx1} = 0.14 \pm 0.02$ μmol P_i/mg per min, $K_{m1} = 17 \pm 4$ μM; (C) $V_{mx2} = 0.60 \pm 0.06$ μmol P_i/mg per min, $K_{m2} = 235 \pm 24$ μM; (D) $V_{mx2} = 0.94 \pm 0.7$ μmol P_i/mg per min, $K_{m2} = 540 \pm 35$ μM.

(broken lines) describes a simple Michaelian relationship between activity and substrate concentration (Eqn. 1), where

$$v = V_{mx} \cdot s/(K_m + s) \quad (1)$$

and the other (solid lines) is the sum of two Michaelians (Eqn. 2), where

$$v = [V_{mx1} \cdot s/(K_{m1} + s)] + [V_{mx2} \cdot s/(K_{m2} + s)] \quad (2)$$

Within the dispersion of the data points, the best fits were obtained in all cases using Eqn. 2 (fitting errors about one-half or less than those given by Eqn. 1). In addition, the departure of the experimental points from the theoretical ones is more conspicuous in the region of low ATP concentrations. Furthermore, the better fit obtained with Eqn. 2 in Fig. 7 is not a consequence of the increased flexibility due to the larger number of fitting parameters (four in Eqn. 2 instead of two in Eqn. 1). This was evidenced by the Snedecor's F test applied ot the differences between experimental and theoretical points, which gave the following results: (A) $F = 9.51$, df = 35 and 33, $P < 0.01$; (B) $F = 4.6$, df = 35 and 33, $P < 0.05$; (C) $F = 53.9$, df = 32 and 30, $P < 0.01$; (D) $F = 11.2$, df = 32 and 30, $P < 0.01$. The component of high-ATP affinity (K_{m1} between 12–16 μM) had the lower V_{mx} (V_{mx1} between 0.13–0.18 μmol P_i/mg per min); on the other hand, that with low-ATP affinity (K_{m2} in the range of 241 μM to 382 μM) had a V_{max} (V_{mx2}) between 0.52 and 0.85 μmol P_i/mg per min. Two main facts are evident from Fig. 7: (i) the best fit to two rather than one Michaelian function observed under the four experimental conditions tested indicates that the overall shape of the ATP activation curve is not determined by the [Mg^{2+}] and/or [K^+]; (ii) despite that, there seems to be a tendency of K_{m2} to increase at high Mg^{2+}, and particularly Mg^{2+} + K^+ concentrations.

The possibility that the concentrations of these cations indeed affect the kinetic parameters for ATP was explored by determining dose-response curves for ATP stimulation at two extreme concentrations of Mg^{2+} and K^+: one, which we could call optimal (1 mM Mg^{2+} and 10 mM K^+), and another where the activity was expected to be largely inhibited at low [ATP] (20 mM Mg^{2+} and 60 mM K^+). Once the fitting to Eqn. 2 was obtained, and after proper subtractions were carried out (see legend to Fig. 8), each component was plotted separately as a function of the [ATP]. Fig. 8 illustrates one of the three experiments performed, where the high(left panels)- and low(right panels)-ATP affinity components appear at optimal (A and C) and inhibitory (B and D) [Mg^{2+} + K^+]. The points were fitted to single Michaelian functions. The mean values, plus minus the S.E., for the kinetic parameters obtained from three different experiments were: (A) $V_{mx1} = 0.28 \pm 0.03$ μmol P_i/mg per min, $K_{m1} = 13 \pm 2$ μM; (B) $V_{mx1} = 0.14 \pm 0.02$ μmol P_i/mg per min, $K_{m1} = 17 \pm 4$ μM; (C) $V_{mx2} = 0.60 \pm 0.06$ μmol P_i/mg per min, $K_{m2} = 235 \pm 24$ μM; (D) $V_{mx2} = 0.94 \pm 0.7$ μmol P_i/mg per min, $K_{m2} = 540 \pm 35$ μM.

Finally, it is important to stress at this point that similar results were obtained in enzyme incubated with 20 mM potassium nitrate and when all chloride was substituted with sulfate (not shown).

Discussion

Despite the fact that we are dealing with a crude plasma membrane preparation, its general characterization as well as the properties of the observed ATPase activity indicate the presence of a plant H-ATPase. Therefore, against the possible argument that the results described here come from a rather crude preparation, we can oppose the advantage that it has not been subjected to any treatment with detergents, something which could result in a more pure, but perhaps also in a modified enzyme.

The complex pattern of ATP-dependent phosphorylation in LDS-PAGE gels suggest that transphosphorylating enzymes other than ATPase are present in these membranes. Kinases are the most likely ones; yet, as all phosphorylations were performed in the absence of ionized Ca^{2+} (0.5 mM EGTA was included) indicate that they do not belong to the class which is Ca^{2+}-dependent [30]. The phosphorylated membrane fraction with an $M_r = 100\,000$, which almost surely represents the phosphointermediate of the ATPase reaction, has shown equal sensitivity to both K^+ and ADP. Our approach does not allow to tell whether we are in the presence of one or more E-P forms. If there is only one, then that is likewise attacked by both ligands; nevertheless, similar results would be obtained if, as suggested by Briskin in *Beta vulgaris* L. [13,14], two phosphoenzymes, one sensitive to ADP and the other to K^+, can be rapidly interconverted.

It would be highly desirable that the dual effects of Mg^{2+} and K^+ (activation at low and inhibition at high concentrations), the antagonism between ATP and the inhibition by Mg^{2+}, K^+ or Mg^{2+} + K^+, and the complex ATP activation curves, could be explained by a single kinetic scheme. An interesting possibility is the existence of two ATP sites per active enzyme molecule. Evidences for this situation, based on competitive binding of ATP and a fluorescent analogue, have been reported for the gastric H,K-ATPase of mammals [31]. Under this conception there are alternatives: (i) one site is catalytic and the other regulatory, and (ii) the two are catalytic. Alternative (i) means a H-ATPase cycle where a dephosphoenzyme refractive to phosphorylation from ATP is transformed into a form susceptible to such phosphorylation; that transformation is accelerated by

the nucleotide acting with low affinity in a regulatory role. This hypothesis is not inconceivable on the light of the ATP stimulation curves of hydrolysis in Fig. 7 which cannot be fitted to a single Michaelian function. The additional requirement that K^+ and Mg^{2+} behave as 'product' inhibitors favoring the dephosphoenzyme form refractive to ATP (see Ref. 32 for the Na,K-ATPase) can account for the ATP-(Mg^{2+} + K^+) antagonism (described in Figs. 3–6 and 8). Furthermore, this scheme is compatible with a single site for each of the aforementioned cations which changes its affinity during the cycle depending on the enzyme conformation. Alternative (ii), that is two catalytic (phosphorylating) loci for ATP, would also give ATP activation curves of the type observed in Fig. 7. In this case, the Mg^{2+}, K^+ and ATP interactions shown here should be different at each phosphorylation site. Incidentally, other important information can be extracted from Fig. 8: on the one hand, K^+ stimulation of ATP hydrolysis can be detected even at the so called 'inhibitory' [Mg^{2+}] and [K^+] provided [ATP] is not limiting; on the other, Mg^{2+} and K^+ may also affect the high-ATP affinity component (V_{mx1} in this case). In this regard it should be pointed out that in the Na,K-ATPase high [Mg^{2+}] reduces the V_{mx1} and increases the K_{m1} [32,33], an indication of the complexity of Mg^{2+}–enzyme interactions.

The results of Fig. 7 may obey to reasons other than two ATP sites in the enzyme. For instance, in a crude preparation like ours, the coexistence of two ATPase enzymes with different affinities for the substrate is possible. The unequal V_{max} values would reflex unequal turnover numbers or units abundance. Yet, both the high- and low-ATP affinity components have the same pH dependence and are inhibited by vanadate, diethylstilbestrol and erythrosine (not shown); accordingly, if there are two enzymes both are likely to be H-ATPases. The presence of two enzymes due to contamination from structures other than plasma membrane (tonoplast for instances) does not seem very likely because similar behavior was observed in the presence of 20 mM nitrate and in chloride free media (not shown). An attracting possibility is that there are two isoforms of the plasma membrane H-ATPase. The existence of isoforms for this enzyme has been suggested by Pardo and Serrano [34] on the basis of cDNA sequence studies. Obviously, if this is the explanation, the two isoforms must react differently with Mg^{2+}, K^+ and ATP.

Finally, the fitting analysis reveals a large difference between K_{m1} and K_{m2} even under conditions where Mg^{2+} and/or K^+ inhibition are not detected. Therefore, for a two-ATP site (one regulatory) model in plants H-ATPase, the transition from an ATP refractive to a receptive form must be intrinsically slow at low [ATP], regardless the [Mg^{2+}] and the presence or absence of K^+.

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