

Mechanistic Investigation on the Temperature Dependence and Inhibition of Corn Root Plasma Membrane ATPase

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The kinetics of corn root plasma membrane-catalyzed Mg-ATP hydrolysis may be satisfactorily described by a simple Michaelis-Menten scheme. It was found that the K_m of the process was relatively insensitive to changes in temperature. This property allowed us to conveniently estimate the activation energy of the enzyme turnover process as ~ 14 kcal mol⁻¹ in the temperature range of 10 to 45°C. The enzyme activity was inhibited by the presence of diethylstilbestrol (DES), miconazole, vanadate, and dicyclohexylcarbodiimide (DCCD). The inhibition caused by DES and miconazole was strictly uncompetitive and inhibition by vanadate was noncompetitive. The inhibition by DCCD showed a substrate concentration dependence, i.e., competitive at high and uncompetitive at low concentrations of Mg-ATP. The $1/V$ vs $[I]$ plots suggested that there were different but unique binding sites for DES, vanadate, and miconazole. However, the modification of the plasma membrane by DCCD exhibited interaction with multiple sites. Unlike yeast plasma membrane ATPase, the enzyme of corn root cells was not affected by the treatment with *N*-ethylmaleimide. Although the enzyme activity was regulated by ADP, a product of the reaction, the presence of inorganic phosphate showed no inhibition to the hydrolysis of Mg-ATP. © 1985 Academic Press, Inc.

The plasma membrane of plant root cells contains an ATPase system which utilizes Mg-ATP as its preferred substrate (1-4). The hydrolysis of ATP catalyzed by this membrane enzyme followed a simple Michaelis-Menten scheme (5-7). The properties of this enzyme, in many regards, are similar to that of fungal plasma membrane H⁺-ATPase (4). Indeed, both the fungal (8, 9) and plant root (7, 10-12) plasma membrane ATPases contain a 100-kDa polypeptide which forms a phosphorylated intermediate in the process of catalyzing the hydrolysis of ATP. Furthermore, the highly purified fungal plasma membrane ATPase (13) and the partially

purified root plasma ATPase (14) have been reconstituted into phospholipid vesicles. The reconstituted systems exhibit an ATP hydrolysis-supported electrogenic proton-pumping activity. Thus, it is likely that plant root plasma membrane ATPase may play a central role in the transport processes occurring between the soil-root interface (1).

Unlike the F_0F_1 type of ATPases found in mitochondria, chloroplasts, and bacteria, the fungal and plant root plasma membrane ATPases are insensitive to the presence of oligomycin (5, 15). These plasma membrane ATPases share striking similarities with E_1E_2 -type ion-translocating ATPases found in animal cells (4, 16). In a previous report (17), we demonstrated that the hydrolysis rate of ATP catalyzed by corn root plasma membrane can be decreased by the presence of ADP, a product of the reaction. In addition, the

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presence of ADP also causes the root plasma membrane ATPase to be less sensitive to the inhibition induced by a wide range of covalent and noncovalent modifiers. These results lead us to propose that in addition to exhibiting product inhibition, the binding of ADP to the membrane may also stabilize the conformation of the active site of the ATPase.

In the present study, we investigated the temperature dependence of the ATPase activity and compared the mode of inhibition by different modifiers which included DES,⁴ miconazole, vanadate, and DCCD. In addition, we found that *N*-ethylmaleimide (NEM) and phosphate did not inhibit the ATPase of corn root plasma membrane. The data also suggested that DES, miconazole, and vanadate were bound to specific sites on the membrane.

MATERIALS AND METHODS

Materials

Oligomycin, DES, miconazole, ATP, sucrose (RNAase-free), malachite green, NADH, phosphoenolpyruvate, crude pyruvate kinase (mixture of pyruvate kinase and lactate dehydrogenase), 4-morpholineethanesulfonic acid (Mes), dithioerythritol (DTE), and 4-(2-hydroxyethyl)-1-piperazineethanesulfonate (Hepes) were obtained from Sigma Chemical Company.⁵ DCCD of gold-label grade was purchased from Aldrich. All other chemicals used were of analytical grade.

Isolation of Plasma Membrane

Plasma membrane-enriched microsomal fraction was isolated from corn roots according to a procedure described by Leonard and Hotchkiss (18) with minor modifications. Untreated corn seeds (FRB-73, Illinois Foundation Seeds) were germinated over filter paper saturated with 0.1 mM CaCl₂ in glass trays for 3 days at 28°C in an Isotemp 300 series incubator. The roots were homogenized in an ice-cold solution

containing 0.30 M sucrose, 5 mM EDTA, 10 mM DTE, 0.1 M Hepes, pH 7.75. The crude microsomal pellet was dispersed with a glass rod and then resuspended in a small volume (1–2 ml) of the solution mentioned above. The microsomal suspension was layered over a discontinuous sucrose gradient (22 ml of 34%, 14 ml of 45%) and centrifuged at 25,000 rpm for 2 h, and the plasma membrane fraction was collected at the interface. All the isolation procedures were performed at 4°C. The protein concentration was determined by the Biuret method using dried bovine serum albumin as the standard. The plasma membrane fraction was frozen in liquid nitrogen and stored at –20°C until use.

Hydrolysis of ATP

Two methods were used to measure the rate of ATP hydrolysis catalyzed by the plasma membrane fraction.

A. Direct determination of inorganic phosphate. The hydrolysis of ATP under various conditions was measured by the appearance of inorganic phosphate in 1 ml of a solution containing 30 mM Mes, pH 6.2, 50 mM KCl, 5 mM MgCl₂, 50–75 µg of membrane protein, 80 µg/mg oligomycin, and other additions as detailed in figures and tables. The reaction was started by the addition of ATP and terminated by the addition of 1 ml of ice-cold 5% trichloroacetic acid at appropriate time intervals. The amount of inorganic phosphate released was determined by the malachite green–molybdate complex method as previously described (19). The time intervals selected ensured that less than 2% of the ATP was hydrolyzed.

B. Coupled enzymatic NADH oxidation. Alternatively, the initial rate of ATP hydrolysis was determined by a coupled enzyme system which consisted of pyruvate kinase and lactate dehydrogenase (20). An appropriate amount of ATP was added to 3 ml of a solution which contained 50 µg plasma membrane protein, 4 µg oligomycin, 54 units of pyruvate kinase, 75 units of lactate dehydrogenase, 0.4 mM phosphoenolpyruvate, 0.3 mM NADH, 5 mM MgCl₂, 30 mM Mes, pH 6.2, and other additions as described in figure legends.

Modification of Plasma Membrane by NEM

Root plasma membrane preparation containing ~2 mg of protein was suspended in 4 ml solution of 20 mM Tris, pH 8.0, and 0 to 10 mM NEM at 0°C for 0 to 1 h. At the end of incubation ~100 µg of treated membrane was transferred to 2 ml of the ATPase assaying solution mentioned, and the catalyzed inorganic phosphate release was measured. The addition of treated membrane to the assaying solution which has a pH value of 6.2 was sufficient to stop the alkylation of –SH group (16).

⁴ Abbreviations used: DES, diethylstilbestrol; DCCD, *N,N*-dicyclohexylcarbodiimide; NEM, *N*-ethylmaleimide; MES, 4-morpholineethanesulfonic acid; DTE, dithioerythritol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

⁵ Reference to brand or firm name does not constitute endorsement by the U. S. Department of Agriculture over others of a similar nature not mentioned.

RESULTS

Temperature Dependence of ATPase Activity

The ATPase activity of root plasma membrane used in this study was not inhibited by oligomycin (80 $\mu\text{g}/\text{mg}$ protein), indicating the absence of F_0F_1 mitochondrial ATPase activity under the experimental conditions. Furthermore, the total ATPase activity of the membrane, when assayed in a solution also containing 5 mM azide, 100 mM KNO_3 , and 0.1 mM molybdate, showed no more than a 10% decrease. This observation suggested a minimal contamination of F_1 mitochondrial, tonoplast, and other nonspecific ATPases (4, 21, 22). It has been demonstrated that the ATPase activity apparently obeys a simple Michaelis-Menten kinetics (5-7). In order to understand the effects of temperature on the kinetic parameters of root membrane ATPase, experiments described in Fig. 1 and Table I were performed.

As shown in Fig. 1A, the catalyzed ATP hydrolysis rate shows a sigmoidal increase as the temperature increased from 5 to

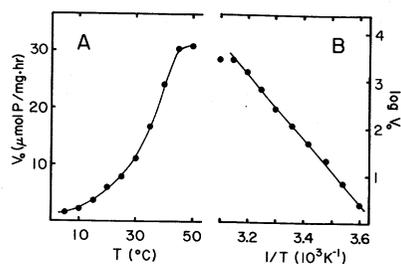


FIG. 1. (A) Temperature dependence of corn root plasma membrane ATPase activity. The ATPase activity of the membrane preparation was assayed at controlled temperature. The reaction rate was determined by coupled enzyme system as mentioned under Materials and Methods. The concentration of ATP used was 3.3 mM. Similar results were also obtained when phosphate was determined directly. However, in the latter process, the extent of ATP hydrolysis was controlled by varying the time interval between the additions of substrate and quenching solution, TCA. (B) Determination of the activation energy associated with the turnover of the enzyme; the Arrhenius plot was obtained with the data mentioned in (A). The detailed justification for this treatment was mentioned in text.

TABLE I

TEMPERATURE DEPENDENCE OF K_m	
T (°C)	K_m^a (mM)
15	0.22 \pm 10%
25	0.20 \pm 10%
35	0.20 \pm 10%

^a K_m was determined by both direct P_i determination and coupled enzyme assay. The results obtained were similar with these two methods. The data shown were the averages of two independent measurements.

$\sim 45^\circ\text{C}$. Above 45°C , the enzyme activity reaches a plateau value. In terms of a simple Michaelis-Menten scheme, such a change may be attributed to a decrease of K_m , an increase of enzyme turnover, or both, at higher temperatures. These possibilities were differentiated by analyzing the kinetic data obtained at various temperatures. As shown in Table I, an increase in temperature does not significantly change the K_m of the reaction. Thus, the observed results shown in Fig. 1A should be attributed to an accelerated enzyme turnover at elevated temperatures. Since, experimentally, the enzyme concentration was kept as a constant, the insensitivity of K_m allowed us to evaluate the activation energy (E_a) of the turnover process with a single saturated ATP concentration. According to simple Michaelis-Menten kinetics, the initial forward reaction rate (V_0), is related to kinetic parameters as

$$V_0 = \frac{k_2[E]_0[S]}{K_m + [S]}, \quad [1]$$

in which k_2 , $[E]_0$, $[S]$ represent turnover rate constant, total enzyme concentration, and initial substrate concentration, respectively. By keeping $[S]$ and $[E]_0$ at constant levels, V_0 in a temperature range may be represented as

$$V_0 = k_2 \times \text{"constant"} \quad [2]$$

if K_m is independent of temperature. Thus, for the plasma membrane ATPase activity

the Arrhenius equation may be incorporated into Eq. [2] as

$$\ln V_0 = -\frac{E_a}{R} \cdot \frac{1}{T} + \ln A + \ln \text{"constant"}, \quad [3]$$

in which A is the normal frequency factor. The activation energy of the enzyme turnover process can then be determined from the slope of $\ln V_0$ vs $1/T$. As shown in Fig. 1B, the mentioned assumption is indeed valid in the temperature range of 5 to 45°C and the activation energy of the turnover process has a value of 14 kcal/mol. The possible implication of this result on the enzyme reaction mechanism will be discussed later.

Comparison of Inhibition

In general, inhibitors can be powerful tools to investigate and elucidate the function and molecular mechanisms of an enzyme. While there are many reports which describe the apparent inhibition of root plasma membrane ATPase by many reagents (7, 15, 21), detailed classification of inhibitors in terms of kinetic modes are not available. In the present study we compared the modes of action of DES, miconazole, vanadate, and DCCD. The effective concentration ranges of these inhibitors were already established in our previous report (19).

A. DES and miconazole. The presence of DES or miconazole significantly inhibited root plasma membrane ATPase activity. The Lineweaver-Burk plot ($1/V$ vs $1/[S]$) shown in Fig. 2A indicates that DES is an uncompetitive inhibitor to the activity of root plasma membrane ATPase. A similar inhibition pattern was also observed for miconazole (Fig. 3A). At 25°C, the binding constants (K_i) of DES and miconazole were determined to be $6.4 \pm 0.3 \times 10^{-5}$ M and $2.2 \pm 0.2 \times 10^{-4}$ M, respectively. Miconazole has been shown to act as a competitive inhibitor to the plasma membrane ATPase of a fungus, *Schizosaccharomyces pombe* (23). However, in the root membrane system this compound and

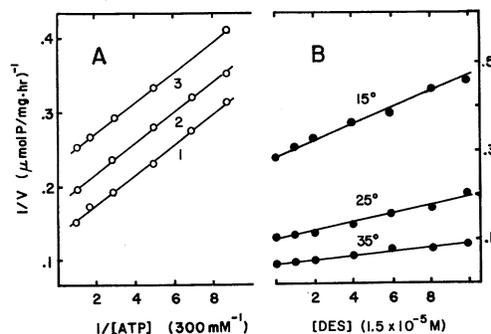


FIG. 2. Diethylstilbestrol inhibition of corn root plasma membrane ATPase. (A) Lineweaver-Burk plots. The ATPase activity was assayed by the direct phosphate determination method. The membrane was treated with the inhibitor for 10 min in the assaying medium before the addition of ATP at 25°C. Traces 1, 2, and 3 represent the ATPase activity in the presence of 0, 2.0, and 5.0×10^{-5} M DES. (B) Dixon plots. The saturated ATPase activity in the presence of 3.3 mM ATP was measured in the presence of different concentrations of DES at different temperatures.

DES both act as uncompetitive inhibitors. The difference indicates that the mechanism of interaction of miconazole to plant root ATPase and fungal plasma membrane ATPase are different.

When the graphical method of Dixon ($1/V$ vs $[I]$) was used to analyze the inhibition under fixed ATP concentrations, a linear relationship was obtained for both miconazole and DES. As shown in Figs. 2B and 3B, this linear relationship holds in a wide temperature range (15–35°C). This result suggests that the membrane contains unique receptor sites for DES and miconazole, respectively. Since the chemical structure of DES is quite different from that of miconazole, it is not expected that the same sites are shared for the binding of both inhibitors.

B. Vanadate. Orthovanadate is a potent inhibitor of the Na^+/K^+ - and Ca^{+2} -dependent ATPases of animal and human cells (24, 25). Plasma membrane-bound ATPases of fungal (26, 27) and plant root systems (21) are also sensitive to this inorganic anion. It has been shown that vanadate acts as a noncompetitive inhib-

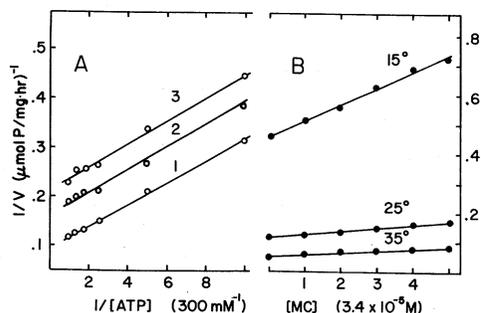


FIG. 3. Miconazole inhibition of corn root plasma membrane ATPase activity. (A) Lineweaver-Burk plots. The general experimental procedure was the same as mentioned in Fig. 2A. Traces 1, 2, and 3 were obtained in the presence of 0, 1.5, and 3.0×10^{-4} M miconazole at 25°C. (B) Dixon plots. The details of experimental conditions were mentioned in Fig. 2B.

itor to plasma membrane ATPase of *S. pombe* (23). As shown in Fig. 4A, vanadate inhibition of root plasma membrane ATPase is also noncompetitive, in agreement with a previous finding (21). Unlike uncompetitive inhibitors which presumably only involve effective binding to enzyme-substrate complex, noncompetitive inhibitors show effective binding to both free and substrate-bound enzymes. Since there was no basis to assume that the binding constants for vanadate of two different forms of the enzyme were the same, no attempts were made to evaluate these constants from the data mentioned in Fig. 4A.

The effective binding of vanadate to root plasma membrane was evaluated by Dixon plots at various temperatures. As shown in Fig. 4B, only one or one set of equivalent binding sites is involved at 25 and 35°C. At lower temperatures, e.g., 15°C, the binding was more complex and certain negative cooperativity, in terms of inhibition efficiency, became evident. Unlike the hydrophobic inhibitors, DES and miconazole, the interaction sites of vanadate ion should be limited to the surface of root plasma membrane. Based on the temperature dependence of NADH-linked functions, it has been suggested that corn root plasma membrane may

show a phase transition below 9°C (28). It is possible that such a transition may affect the surface properties of the membrane and induce a change in the binding of vanadate.

C. Dicyclohexylcarbodiimide. DCCD is a potent inhibitor of mitochondrial, chloroplast, and bacterial H^+ -ATPases which binds to a proteolipid component of the membrane-bound forms of these enzymes (29). It was previously reported that fungal plasma membrane ATPase was also sensitive to DCCD treatment (23), and that the inhibition pattern was strictly noncompetitive in nature. When DCCD was used to treat root plasma membrane, a rather complex inhibition of the ATPase activity was observed. As shown in Fig. 5A, DCCD exhibits noncompetitive inhibition at low substrate concentrations; however, at higher concentrations of ATP the inhibition becomes less effective, and the pattern changes to competitive. The origin of this substrate concentration dependence was not determined. A possible explanation involving conformational interactions between DCCD binding sites and the catalytic site is given under Discussion. The effective binding of DCCD to the membrane was analyzed and the results are shown in Fig. 5B. The data

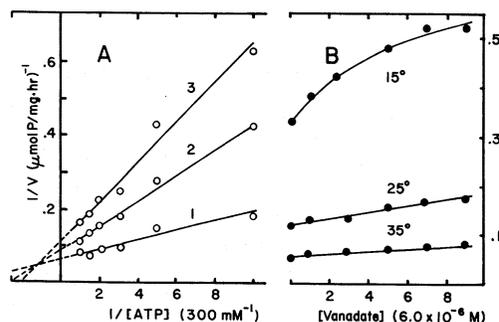


FIG. 4. Vanadate inhibition of corn root plasma membrane ATPase activity. (A) Lineweaver-Burk plots. The general experimental procedure was the same as that mentioned in Fig. 2A. Traces 1, 2, and 3 were obtained in the presence of 0, 1.0, and 5.0×10^{-5} M vanadate at 25°C. (B) Dixon plots. The general experimental conditions were identical to those mentioned in Fig. 2B.

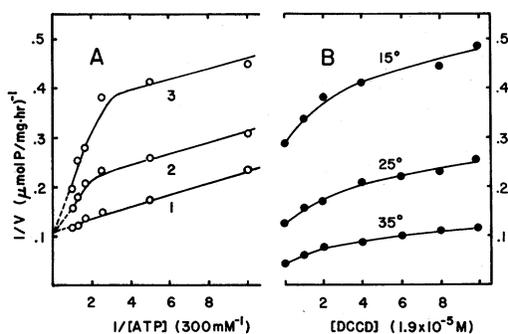


FIG. 5. DCCD inhibition of corn root plasma membrane ATPase activity. (A) Lineweaver-Burk plots. The experimental procedure was the same as that mentioned in Fig. 2A. Traces 1, 2, and 3 were obtained with membrane treatment with 0, 2.0, and 5.0×10^{-5} M DCCD. (Membrane protein, $75 \mu\text{g}/\text{run}$.) (B) Dixon plots. The details of experimental conditions were the same as that mentioned in Fig. 2B.

suggest that there are more than one or one set of equivalent sites for effective DCCD binding. It is known that DCCD modifies a specific carboxyl group of the proton channel formed by the proteolipid sector of mitochondrial, chloroplast, and bacterial H^+ -ATPases (29). Whether the root membrane ATPase contains the similar proton channel or not is unknown at the present time. However, it should be noted that root plasma membrane, rather than reconstituted pure ATPase, was used in the present study. Thus, the binding study mentioned in Fig. 5B does not rule out the possibility that only one site in the ATPase is modified by DCCD.

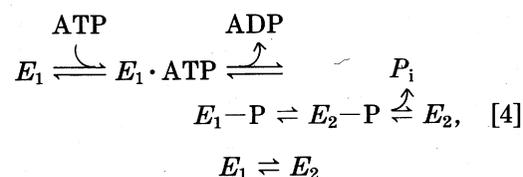
D. NEM and phosphate. It has been demonstrated that the -SH reagent, NEM reacts with fungal plasma membrane ATPase (16, 30). Since the binding of ADP to this enzyme also provides an effective protection against the inhibition of NEM, the authors concluded that there is an essential -SH group at the active site of fungal plasma membrane ATPase. When root plasma membrane was treated with NEM (0 to 10 mM), pH 8.0, for 1 h at 0°C , subsequent ATPase activity measurements did not show any decrease (data not shown). This result indicates that the sensitivity of corn root plasma membrane

ATPase activity is quite different from that of fungal system. However, because of the possible accessibility restriction, the involvement of essential -SH groups may not be excluded completely.

Since inorganic phosphate is also an endproduct of the enzymatic reaction, it is of interest to examine whether the ATPase activity may be affected by this ionic species. No inhibition was found with inorganic phosphate concentrations up to 20 mM (data not shown). This implies that inorganic phosphate binds weakly, if at all, to the ATPase, and that the rate-limiting step of the enzyme reaction occurs before the releasing of inorganic phosphate from the enzyme.

DISCUSSION

Although the exact reaction mechanism of root plasma membrane ATPase is not completely understood at the present time, the existence of a 100-kDa phosphorylated intermediate and inhibition by vanadate, are consistent with the mechanism of a common E_1E_2 type of ATPase (31). A minimal working mechanism for root plasma membrane ATPase may be represented by the following equation



in which “ \cdot ” and “ $-$ ” represent noncovalent and covalent bonds, respectively. The observation that inorganic phosphate does not affect the overall rate of ATP hydrolysis suggests that the rate-limiting step of enzyme turnover cannot be the release of inorganic phosphate from the ATPase. This is in agreement with the observation that the formation of E-P cannot be induced by inorganic phosphate (7). Previously observed ADP inhibition of the enzyme activity (17) may be accounted for by a decrease of the steady-state concen-

tration of phosphorylated intermediate, E-P, through the reverse reaction sequence. It should be mentioned that we assume that the energy states of E·ATP and (E-P + ADP) are not very different. This assumption implies that the energy needed for H⁺ translocation generates from the latter steps of the mechanism.

For the enzyme to turnover, it is essential that an ADP is released and E-P is attacked by other nucleophiles. The observed temperature dependence of the ATPase turnover mentioned in the present report suggests that the rate of either one of these two processes or both increases with increased temperature. The relatively unchanged K_m would indicate that the binding of ATP to root membrane ATPase also increases with temperature to compensate for the faster breakdown of the phosphorylated intermediate.

Since none of the inhibitors tested in this report structurally resembles the substrate of the enzyme, it is unlikely that they would interact with the binding site of Mg-ATP directly. Thus, the observed inhibitory effects are most likely due to conformational changes of root plasma membrane ATPase induced by the presence of inhibitors. Our data indicate that there are specific binding sites for DES, miconazole, and vanadate, respectively, in root plasma membrane. These three inhibitors were also found to have unique binding sites in fungal plasma membrane ATPase (23). However, only the inhibition pattern of vanadate is similar in both membrane systems. The origin of the difference in response of ATPase activity to lipid-soluble inhibitors, DES and miconazole, in root and fungal plasma membrane is not known at the present time. In general, this observation may suggest a difference in membrane composition, essential lipid requirement, or the intramembranous folding of ATPases. The observed substrate concentration dependence of DCCD inhibition in the root membrane system is completely different from that found in fungal system (23). While there are insufficient data to pinpoint the exact cause for this phenomenon,

it is possible to speculate that the root plasma membrane ATPase may, in addition to the catalytic site, also contain another ATP binding site(s) which presumably has a negligible allosteric effect on the catalytic processes. However, the conformational change of the catalytic site caused by DCCD treatment may be modified by the extent of binding of these sites. Thus, a change in the mode of inhibition occurs as the concentration of Mg-ATP increased.

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