

Effects of nucleotide analogs on ATP hydrolysis by P-type ATPases. Comparison between the canine kidney ($\text{Na}^+ + \text{K}^+$) ATPase and maize root H^+ -ATPase

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The mechanism by which chemical energy is converted into an electrochemical gradient by P-type ATPase is not completely understood. The effects of ATP analogs on the canine kidney ($\text{Na}^+ + \text{K}^+$) ATPase were compared to effects of the same analogs on the maize (*Zea mays* L. cv. W7551) root H^+ -ATPase in order to identify probes for the ATP binding site of the maize root enzyme and to determine potential similarities of ATP hydrolysis mechanisms in these two enzymes. Six compounds able to modify the ATP binding site covalently were compared. These compounds could be classed into three distinct groups based on activity. The first group had little or no effect on catalytic activity of either enzyme and included 7-chloro-4-nitrobenz-2-oxa-1,3-diazole. The second group, which included azido adenine analogs, fluorescein isothiocyanate and 5'-*p*-fluorosulfonylbenzoyladenine, were inhibitors of ATP hydrolysis by both enzymes. However, the sensitivity of the ($\text{Na}^+ + \text{K}^+$) ATPase to inhibition was much greater than that exhibited by the maize root enzyme. The third group, which included periodate treated nucleotide derivatives and 2',3'-*o*-(4-benzoylbenzoyl)adenosine triphosphate, inhibited both enzymes similarly. This initial screening of these covalent modifiers indicated that 2',3'-*o*-(4-benzoylbenzoyl)adenosine triphosphate was the optimal covalent modifier of the ATP binding site of the maize root enzyme. Certain reagents were much more effective against the ($\text{Na}^+ + \text{K}^+$) ATPase than the maize root enzyme, possibly indicating differences in the ATP binding and hydrolysis pathway for these two enzymes. Two ATP analogs that are not covalent modifiers were also tested: the trinitrophenyl derivatives of adenine nucleotides were better than 5'-adenylylimidodiphosphate for use as an ATP binding probe.

Key words – 2',3'-*o*-(4-benzoylbenzoyl)adenosine triphosphate, 5'-*p*-fluorosulfonylbenzoyladenine, maize, modification, nucleotide, *Zea mays*.

D. Brauer (corresponding author) and S.-I Tu, Plant and Soil Biophysics Research Unit, Eastern Regional Research Center, ARS, USDA, 600 E. Mermaid Lane, Philadelphia, PA 19118, USA.

Introduction

Enzymes that link transport of ions to either hydrolysis or synthesis of ATP have been classified into three broad categories of transport ATPases: P-, V- and F-type (Nelson and Taiz 1989). These three types of ATPases can be distinguished by their biochemical, physical and physiological properties. Under most physiological conditions, the F-type ATPase couples the flux of either protons or Na^+ down its electrochemical gradient to the synthesis of

ATP. The other two classes of ATPases couple free energy from ATP hydrolysis to the transport of ions against their electrochemical gradient.

Our research group has been investigating the mechanism by which plant V- and P-type ATPases couple energy from ATP hydrolysis to the transmembrane movement of ions. A generalized reaction scheme for the P-type ATPases consistent with a direct coupling mechanism between ATP hydrolysis and ion transport has been proposed (Skou 1965). However, coupling between ATP

hydrolysis and ion transport by P-type ATPases may not be as rigid as initially envisioned (Brauer et al. 1991, Cornelius 1990, Ikemoto et al. 1981, Inesi and deMies 1989).

Regardless of the nature of the coupling of ATP hydrolysis to ion transport, reactions leading to the hydrolysis of ATP are essential to understanding enzymatic catalysis by P-type ATPases. During the reaction mechanism of this class of ATPases, a specific aspartyl carboxyl group becomes phosphorylated by the terminal phosphate from ATP (Briskin 1990). Amino acids on both sides of this aspartate are highly conserved among enzymes within the P-type class of ATPases (Walderhaug et al. 1985), indicating that this region of the enzyme is critical to the reaction mechanism. More detailed studies have indicated that amino acids from other regions of the protein also are involved in ATP binding and hydrolysis (Clarke et al. 1990, Maryama et al. 1989, Vilsen et al. 1991). Since the overall homology in primary structure of the plant P-type ATPase and other P-type ATPases averages only 20 to 36% (Harper et al. 1989), different P-type ATPases may have different parts of the primary structure involved in the ATP hydrolysis pathway. Such a difference is indicated by apparent differences in the sensitivities of different P-type ATPases to ATP analogs. Fluorescein isothiocyanate (FITC) has been utilized to label a specific Lys residue in the ATP binding site of P-type ATPases (Pardo and Slayman 1988). However, the degree of modification and the sensitivity of enzymatic activity to inhibition varies between different ATPases. The ($\text{Na}^+ + \text{K}^+$) ATPase is inhibited by micromolar levels of FITC after a brief exposure (Kirley et al. 1984), whereas millimolar levels are necessary to inhibit the ATPase from red beet plasma membrane (Gildensoph et al. 1987). This relative insensitivity of the plant enzyme to FITC labeling may indicate that the ATP binding and hydrolysis pathway of the plant enzyme differs from those of the ($\text{Na}^+ + \text{K}^+$) ATPase. To explore the ATP utilization pathway of the plant enzyme further, we attempted to identify a specific covalent label for the ATP binding site by comparing the relative effectiveness of a series of compounds on the maize root enzyme and the canine kidney ($\text{Na}^+ + \text{K}^+$) ATPase.

Abbreviations – AMP-PNP, adenylylimidodiphosphate; AMP-TNP, ADP-TNP, ATP-TNP, trinitrophenyl derivatives of adenine nucleotides; BTP, bis-tris-propane; BzATP, 2',3'-*o*-(4-benzoylbenzoyl)adenosine triphosphate; DOC, deoxycholate; FITC, fluorescein isothiocyanate; FSBA, 5'-*p*-fluorosulfonylbenzoyladenine; N_3 -ATP, azido derivative of ATP; NBD-Cl, 7-chloro-4-nitrobenz-2-oxa-1,3-diazol; *o*AMP, *o*ADP, *o*ATP, periodate-treated adenine nucleotides;

Materials and methods

Isolation of plasma membranes

Maize seedlings (*Zea mays* L. cv. W7551) were grown on filter paper moistened with 0.1 mM CaCl_2 (Nagahashi and Baker 1984). Microsomes were isolated from 3-day-

old roots by differential centrifugation as described previously (Brauer et al. 1988), except the 25 mM bis-tris-propane (BTP) in the homogenizing buffer was replaced with 50 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) (pH 7.5). Plasma membranes were purified by discontinuous sucrose density centrifugation (Brauer et al. 1992). Where indicated, 90 to 95% of the membrane lipids were extracted by exposing plasma membranes to 0.6% (w/v) sodium deoxycholate (DOC), with most of the ATPase collected in the particulate material after it was centrifuged at 100 000 g for 2 h at 4°C (Brauer et al. 1989). Protein concentration was determined by a modification of the Lowry protocol using bovine serum albumin as a standard (Bensadoun and Weinstein 1976).

Assays for vanadate-sensitive ATPase activities

ATP hydrolysis by maize root plasma membranes was monitored colorimetrically by the release of inorganic phosphate (Brauer et al. 1989). The assay medium contained 17.5 mM 2-[N-morpholino]ethanesulfonic acid (MES) titrated to pH 6.45 with BTP, 0.2 mM ethylene-glycol-bis(β -aminoethylether-N,N'-tetraacetic acid) (EGTA), 3.0 mM MgSO_4 , 2.5 mM ATP and 50 mM KNO_3 . Vanadate-sensitive ATPase activity was determined by measuring the difference between rates of ATP hydrolysis in the absence and presence of 0.2 mM sodium ortho-vanadate. The assay medium was supplemented with 200 μg phosphatidylglycerol ml^{-1} when DOC-treated membranes were used, which was necessary to restore enzymatic activity to maximum levels, presumably by adding activating lipids removed by detergent treatment (Brauer et al. 1989). Throughout these investigations, plasma membranes had an activity of 320 ± 20 nmol P_i (mg protein) $^{-1}$ min^{-1} under standard assay conditions. DOC-treated membranes had a specific activity of 740 ± 20 nmol P_i (mg protein) $^{-1}$ min^{-1} . Data are the averages of at least two experiments of three to five replicates. Bars indicating the magnitude of the standard error of the mean are included in the figures when the variation exceeds the size of the data symbol.

The rate of ATP hydrolysis by the canine kidney ($\text{Na}^+ + \text{K}^+$) ATPase (Sigma) was also determined by the release of inorganic phosphate (Brauer et al. 1989). The assay media contained 20 mM HEPES (pH 7.5), 100 mM NaCl, 25 mM KCl, 5 mM MgSO_4 , 5 mM ATP and 0.2 mM EGTA. Activity determined by measuring the rate of ATP hydrolysis in the absence and presence of 0.1 mM sodium ortho-vanadate was attributed to the ($\text{Na}^+ + \text{K}^+$) ATPase, which averaged 1400 nmol P_i (mg protein) $^{-1}$ min^{-1} under standard conditions.

In certain experiments it was desirable to assess the effects of an inhibitor in the presence of both enzymes simultaneously. Typically, 100 μl of canine kidney enzyme and 300 μl of maize root enzyme were diluted to a final volume of 500 μl with the balance being made up by the additions of buffers, inhibitors and other reagents as

described in either the legends of tables and figures or the text. By selecting the appropriate assay conditions, it was possible to assess the activities of both enzymes in the mixture. The assay medium contained 17.5 mM MES titrated to pH 6.45 with BTP, 20 mM KNO₃, 60 mM NaCl, 5 mM MgSO₄, and 0.1 mM EGTA. The reaction was initiated by addition of ATP to a final concentration of 5 mM. Assays were performed in the above mixture and in the presence of 0.2 mM ouabain with and without 0.2 mM vanadate. Addition of 0.2 mM ouabain was sufficient to completely inhibit the (Na⁺+K⁺) ATPase without affecting the activity of the maize root enzyme (data not shown). Therefore, the activity sensitive to ouabain alone corresponded to the level of (Na⁺+K⁺) ATPase activity. ATP hydrolysis by the maize root enzyme was determined by the difference between activities in the presence of ouabain without and with vanadate. Validity of the method was tested by comparing rates of ATP hydrolysis by the two enzymes together to that of the sum of the two enzymes assayed separately (data not shown). The specific activity of the canine kidney ATPase was less under these conditions, averaging 700 nmol P_i (mg protein)⁻¹ min⁻¹ because the pH of the assay media was below the enzyme's optimum. The specific activity of the maize root enzyme was similar to that found under the other assay condition, 310 ± 16 nmol P_i (mg protein)⁻¹ min⁻¹.

The substrate, substantially vanadate-free ATP, the periodate-treated adenine nucleotides, 2',3'-*o*-(4-benzoylbenzoyl)adenosine triphosphate (BzATP), 5'-fluorosulfonylbenzoyladenine (FSBA), adenylylimidodiphosphate (AMP-PNP) and azido derivative of ATP (N₃-ATP) were obtained from Sigma (St Louis, MO, USA). The trinitrophenyl derivatives of adenine nucleotides, FITC and 7-chloro-4-nitrobenz-2-oxa-1,3-diazol (NBD-Cl) were obtained from Molecular Probes Inc. (Eugene, OR, USA).

Photolysis

Several of the tested ATP analogs are able to react with the side chains of amino acids to covalently label the presumed ATP binding site in the presence of UV illumination. To photolyze these reagents, the enzyme solution was transferred to a 1-ml quartz cuvette with a 1-cm pathway and the cuvette was sealed with Parafilm. The cuvette was placed on top of a Mineralight lamp such that the 1-cm light path of the cuvette was perpendicular to the light bulb of the lamp (Model UVSL-25, San Gabriel, CA, USA) and illuminated with either short- (254 nm) or long- (365 nm) wavelength UV light. This lamp produces a photon flux of 7.5 and 1.8 W m⁻² of 254 and 365 nm, respectively, at a distance of 7.6 cm. Illumination periods of less than 15 min had little, if any, effect on ATP hydrolysis by either the canine or maize P-type ATPase in the absence of photoactivatable compounds (data not shown). Therefore, both ATPases were fairly resistant to inhibition due to illumination alone.

Results and discussion

FITC inhibition

We began our investigations by studying the utility of FITC as an inhibitor of the maize root P-type ATPase. When plasma membranes from maize roots were incubated with 0.1 mM FITC for 10 min at pH 8.0, the activity of the vanadate-sensitive ATPase decreased to 76 ± 3% of that of membranes incubated with solvent alone (Tab. 1). When the (Na⁺+K⁺) ATPase was treated under similar conditions, ATP hydrolysis decreased to 35 ± 5% of that of controls. Such results suggested that maize root enzyme was less sensitive to FITC-induced inhibition. To substantiate this contention, FITC labeling was conducted on maize root plasma membrane mixed with canine (Na⁺+K⁺) ATPase. After the incubation period, (Na⁺+K⁺) ATPase activity in the mixture declined to 32% of the initial value, while the maize root activity declined only to 75% of that of controls. This marked difference in inhibition persisted when both enzymes were exposed simultaneously to FITC, indicating that there was no soluble agent in the maize root membrane preparations that affected the ability of FITC to inhibit the enzyme. Further evidence that the relative insensitivity of the maize root enzyme to FITC reflected a property of the enzyme came from experiments using substantially delipidated DOC-treated membranes. When DOC-treated membranes were incubated with 0.1 mM FITC for 10 min at pH 8.0, the activity of the vanadate-sensitive ATPase decreased to 78 ± 3% of that of membranes incubated with solvent alone (Tab. 1). This change in activity was not significantly different from the degree of inhibition noted above for untreated membranes. Therefore, removal of most of the lipids, including phosphatidylethanolamine (which contains a primary amine), had no effect on FITC sensitivity. Because the maize root enzyme was rather insensitive to inhibition by FITC, and presumably modification of the ATP binding site, other adenine nucleotide analogs were tested.

Tab. 1. Effects of FITC on ATP hydrolysis (nmol P_i [mg protein]⁻¹ min⁻¹) by the canine kidney and maize root enzymes. Both enzymes were exposed separately or together to 0.1 mM FITC in 20 mM HEPES (pH 8.0) for either a few seconds or 10 min at room temperature prior to assay of ATP hydrolysis activity as described under Materials and methods. In certain experiments, the maize root enzyme had been delipidated by treatment with DOC as described under Materials and methods. Results are the mean ± SE of data from 3 experiments, each with 3 replicates.

Enzyme source	Exposure to FITC	
	0 min	10 min
Canine kidney alone	1370 ± 42	480 ± 26
Maize root alone	310 ± 10	240 ± 10
Mixture		
Canine kidney component	710 ± 36	230 ± 10
Maize root component	300 ± 12	230 ± 12
DOC-trt maize root	730 ± 29	570 ± 17

Tab. 2. Effects of 0.5 mM dialdehyde derivatives of adenosine nucleotides (*o*AMP, *o*ADP and *o*ATP) on the ATP hydrolysis (nmol P_i [mg protein]⁻¹ min⁻¹) by canine kidney and maize root P-type ATPase.

Inhibitor	Enzyme source	
	Maize root	Canine kidney
Control	310 ± 10	1410 ± 30
0.5 mM <i>o</i> AMP	280 ± 14	1370 ± 69
0.5 mM <i>o</i> ADP	220 ± 14	1390 ± 72
0.5 mM <i>o</i> ATP	155 ± 13	1360 ± 92

Periodate-treated nucleotide derivatives

Treatment of nucleotides with periodate results in formation of a dialdehyde derivative, which can bind to the ATP-binding site and slowly undergo Schiff base reaction with available side chains (Bidwai et al. 1989). The plasma membrane ATPase from *Neurospora crassa* has been shown to be inactivated by periodate derivatives of adenosine nucleotides in a manner consistent with covalent labeling near the ATP binding site (Bidwai et al. 1989). Initially, maize root plasma membranes and canine kidney (Na⁺ + K⁺) ATPase were assayed in the presence of 5 mM *o*AMP, *o*ADP or *o*ATP to determine if the presence of these derivatives was inhibitory without covalent modification. The presence of *o*AMP, *o*ADP and *o*ATP decreased ATP hydrolysis to 90, 72 and 50% of control values, respectively (Tab. 2). Similar results were found when the derivatives were reduced with NaBH₄ (data not shown). No such inhibition was observed with the (Na⁺ + K⁺) ATPase (data not shown).

The addition of any of the three dialdehyde derivatives to the maize root plasma membranes increased the apparent vanadate-insensitive ATP hydrolysis (data not shown). This increase stemmed from two factors. First, each of the dialdehyde preparations contained significant amounts of phosphate as a contaminant. Second, there was a time-dependent, vanadate-insensitive increase in phosphate release in the presence of each of the derivatives, apparently associated with hydrolysis of the derivative (data not shown) by phosphatase activity other than that of the H⁺-ATPase. The magnitude of hydrolysis increased with the number of phosphates on the adenosine moiety (data not shown). Therefore, the estimates of vanadate-sensitive ATP hydrolysis by the P-type ATPase of maize root plasma membranes were less reliable in the presence of *o*ATP than of *o*ADP or *o*AMP (Tab. 2).

To assess if *o*ATP could inhibit the maize root ATPase by covalent modification, maize root membranes were incubated 30 min at 22°C with 10 mM *o*ATP. Incubation was terminated by diluting the mixture with 10 volumes of resuspension buffer and the membranes were collected after centrifugation of the suspension at 90 000 g for 60 min at 4°C. Membranes treated in the above manner had a level of ATPase activity that was 78% that of membranes immediately diluted and centrifuged after the ad-

dition of *o*ATP. In comparison, the activity of (Na⁺ + K⁺) ATPase decreased to a similar value after a 30-min exposure to 10 mM *o*ATP (data not shown). Therefore, a similar level of inhibition was found with *o*ATP when the two enzymes were compared. The use of *o*ATP as a covalent modifier of the ATP binding on the maize root P-type ATPase was not pursued further because of questions concerning the specificity of the interaction between *o*ATP and the proton pump. The concentration and length of incubation with *o*ATP necessary for inhibition were quite extreme, suggesting that the specificity of the interaction was rather low and possibly not at the substrate site for ATP. Previously, Schrou and Post (1989) reported that modification of the (Na⁺ + K⁺) ATPase by millimolar levels of *o*ATP did not label the ATP substrate site. In addition, the continual hydrolysis of the reagent *o*ATP and concomitant change in *o*ATP concentration would have made estimates of the inhibition constants and other kinetic parameters difficult.

Effects of modification by fluorosulfonylbenzoyladenine (FSBA)

FSBA, an alkylating compound with a structure similar to ATP, appears to react with Ser, Tyr, Lys and His residues involved in ATP binding or hydrolysis (Colman et al. 1977). As with other P-type ATPases, residues alkylated by FSBA are not near to the Asp that becomes phosphorylated or the Lys modified by FITC (Ohta et al. 1986). Incubation of (Na⁺ + K⁺) ATPase with 5 mM FSBA resulted in a time-dependent decrease in ATP hydrolysis (Fig. 1), such that negligible activity remained after 10 min of incubation. Relatively long incubations, however, were necessary to reduce the activity of the maize root P-type ATPase. Thus, there appeared to be large differences in the sensitivity of these two enzymes to FSBA. A mixing experiment was conducted to assess the possibility that some factor in the maize root enzyme preparations interfered with the ability of FSBA to interact with proteins (Tab. 3). Only 15 ± 2% of the initial (Na⁺ + K⁺) ATPase activity remained compared to 85 ± 3% of the initial maize root activity, when the (Na⁺ + K⁺) ATPase and maize root ATPase were incubated together with 5 mM FSBA for 10 min. Because the inhibition of the (Na⁺ + K⁺) ATPase in the presence of the maize root enzyme was similar to that found in its absence (Tab. 3), the ability of FSBA to react was not deleteriously affected by constituents in the maize root preparation. Sensitivity of the maize root enzyme in DOC-treated membrane preparations was not significantly different from that of untreated membranes (Tab. 3), indicating that the presence of membrane lipids and structure did not account for differences in enzyme sensitivity to FSBA.

Effects of N₃-ATP modification

The azido-derivatives of ATP (N₃-ATP) have been used to label the ATP binding site on a variety of enzymes that

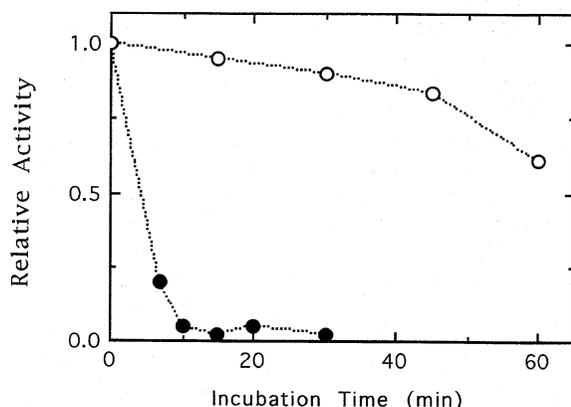


Fig. 1. Effects of incubation with FSBA. Preparations of canine kidney ($\text{Na}^+ + \text{K}^+$) ATPase (●) and maize root plasma membranes (○) were incubated with 5 mM FSBA for 0 to 30 min at 0 to 4°C. After incubation, the enzyme preparations were diluted at least 20-fold with media and assayed immediately for ATP hydrolysis as described in Materials and methods. Data are plotted relative to the activity of enzyme preparations exposed to the FSBA and immediately diluted with assay media, which averaged 1390 ± 22 and 330 ± 9 nmol P_i (mg protein) $^{-1}$ min $^{-1}$ for canine and maize root enzyme, respectively.

use ATP (Schoner and Scheiner-Bobis 1988). Upon short wavelength UV illumination, a nitrene group is formed, which is able to insert into C–H bonds. The potential of this possible site-specific covalent label was explored. In the case of the canine kidney enzyme, photolysis of the N_3 -ATP must be done in the presence of Mg for efficient inhibition (Tab. 4), as was found with ($\text{Na}^+ + \text{K}^+$) ATPases from other sources (Schoner and Scheiner-Bobis 1988). A 10-min illumination at 254 nm in the presence of 10 mM N_3 -ATP and 20 mM MgSO_4 resulted in a 90% reduction of ATP hydrolysis by the ($\text{Na}^+ + \text{K}^+$) ATPase. Illumination of the maize root enzyme under similar conditions resulted in less inhibition, only a 30 to 40% decrease in activity. In contrast to the situation in canine enzyme, the presence of Mg^{2+} did not alter the degree of inhibition (Tab. 4). The low reactivity between N_3 -ATP and the

Tab. 3. Effects of incubation in the presence of FSBA on ATP hydrolysis (nmol P_i [mg protein] $^{-1}$ min $^{-1}$) by the canine kidney and maize root P-type ATPase. Preparations of canine kidney or maize root P-type ATPase were incubated separately or together in the presence of 5 mM FSBA for 10 min at room temperature before ATP hydrolysis was analyzed as described under Materials and methods. Results are the average \pm SE of data from 2 experiment each with 3 replicates.

Enzyme source	Incubation with FSBA	
	0 min	10 min
Canine kidney	1380 ± 28	210 ± 10
Maize root PM	330 ± 7	280 ± 8
Mixture		
Canine	710 ± 18	110 ± 4
Maize root	330 ± 8	290 ± 12
DOC-trt maize root PM	740 ± 21	630 ± 25

maize root enzyme discouraged our use of this reagent as a probe of the ATP binding site.

Effects of NBD-Cl

The reagent NBD-Cl is a chemical modifier of Cys, Tyr and Lys. Under the appropriate conditions NBD-Cl reacts near the ATP binding site of enzymes (Ferguson et al. 1975). The V-type ATPase from plants has been found to be susceptible to this reagent (Randall and Sze 1986). A 15-min exposure of either canine or maize enzyme to 10 mM NBD-Cl in either the absence or presence of Mg^{2+} had a negligible effect on either of the P-type ATPases tested (data not shown), suggesting that there is a fundamental difference in ATP binding by V- and P-type ATPase.

Effects of BzATP

Another analog used to label ATP binding sites is BzATP (Manolson et al. 1985). As with azido derivatives, UV illumination catalyzes the covalent modifications of amino acids that comprise the ATP binding site. Illumination of the ($\text{Na}^+ + \text{K}^+$) ATPase with long wavelength UV light for 10 min in the presence of 1 mM BzATP and 1 mM MgSO_4 resulted in a 45% decrease in activity. Inhibition of ATP hydrolysis was dependent upon UV illumination (data not shown) and was stimulated by the addition of Mg^{2+} (Tab. 5), indicative of inactivation at the catalytic site for ATP hydrolysis. Treatment of the maize root enzyme under similar conditions resulted in a comparable decrease in ATP hydrolysis. Addition of either 2 mM ATP or 0.2 mM vanadate during illumination prevented inhibition induced by BzATP. Since these reagents, ATP and vanadate, interacted with the ATPase at the catalytic site for ATP hydrolysis (Macara 1980), the reduction in inhibition strongly suggested that BzATP exerted its effects at or near the ATP catalytic site.

Tab. 4. Effects of photolysis of N_3 -ATP derivative on the ATP hydrolysis by canine kidney and maize root P-type ATPase. Preparations of the two P-type ATPases were incubated in the presence of 10 mM N_3 -ATP in the absence and presence of 20 mM MgSO_4 , exposed to 254 nm light for 10 min and assayed for ATP hydrolysis as described under Materials and methods. Data are the average \pm SE of 3 experiments each with 3 replicates and presented relative to activity of enzyme exposed to 254 nm light in the absence of N_3 -ATP. The specific activity of such controls averaged 1380 ± 21 and 320 ± 8 nmol P_i (mg protein) $^{-1}$ min $^{-1}$ for the canine kidney and maize root enzyme, respectively.

Enzyme source	Activity after photolysis	
	– MgSO_4	+ MgSO_4
Canine kidney	0.82 ± 0.04	0.09 ± 0.02
Maize root plasma membrane	0.68 ± 0.05	0.62 ± 0.04

Tab. 5. Effect of photolysis of BzATP on ATP hydrolysis by canine and maize root P-type ATPase. Both enzyme preparations were exposed to 10 min of 366 nm illumination in the presence of the reagent listed below. Data are presented relative to enzyme illuminated in the absence of additions and represent the average of 2 experiments each with 3 replicates.

Conditions of illumination	Canine kidney	Maize root
1 mM BzATP	0.83 ± 0.02	0.85 ± 0.04
1 mM BzATP and 1 mM MgSO ₄	0.55 ± 0.02	0.52 ± 0.03
1 mM BzATP, 1 mM MgSO ₄ , 1 mM ATP	0.94 ± 0.02	0.96 ± 0.02
1 mM BzATP, 1 mM MgSO ₄ , 0.2 mM vanadate	0.95 ± 0.02	0.97 ± 0.04

Non covalent modifiers

Classes of ATP derivatives that cannot covalently modify the protein have been used to probe the ATP binding site of P-type ATPase. The most noted derivatives are the trinitrophenyl derivatives of AMP (AMP-TNP), ADP (ADP-TNP) and ATP (ATP-TNP). These derivatives undergo a change in their fluorescent properties upon binding to the ATPase (Moczydlowski and Fortes 1981) and therefore can be used to study the partial reactions involving ATP binding to the enzyme. Addition of 0.2 mM AMP-TNP, ADP-TNP or ATP-TNP to the reaction mixture for measuring ATP hydrolysis by the (Na⁺ + K⁺) ATPase containing 2.0 mM ATP resulted in a decrease in activity (Tab. 6). The AMP derivative was not as effective an inhibitor as either ADP or ATP derivatives. When similar experiments were conducted with the maize root enzyme, the pattern of inhibition differed. The AMP derivative was the most effective inhibitor, and the level of inhibition with AMP-TNP was greater than that observed with the (Na⁺ + K⁺) ATPase.

Differences in effectiveness of ADP-TNP and ATP-TNP as inhibitors of ATP hydrolysis between the canine and maize ATPases may not reflect a difference in the interaction between these derivatives and the enzymes. With the maize root enzyme preparation, there was sub-

Tab. 6. Effects of various trinitrophenyl adenosine derivatives on ATP hydrolysis by maize root and canine kidney P-type ATPases. ATP hydrolysis by the maize root and canine kidney P-type ATPase was measured in the presence of 0.2 mM levels of AMP-TNP, ADP-TNP or ATP-TNP. Data are presented relative to the activity in the presence of 2 mM ATP and are the average ± SE of 4 experiments with 3 replicates each (n=12), which averaged 1380 ± 18 and 330 ± 7 nmol P_i (mg protein)⁻¹ min⁻¹ for canine and maize enzyme, respectively.

Substrate	Relative activity	
	Canine kidney	Maize root
AMP-TNP	0.39 ± 0.01	0.24 ± 0.02
ADP-TNP	0.19 ± 0.02	0.40 ± 0.04
ATP-TNP	0.19 ± 0.02	0.44 ± 0.05

stantial hydrolytic release of inorganic phosphate from ADP-TNP and ATP-TNP (data not shown). This hydrolysis was not vanadate-sensitive, and probably resulted from enzymes other than the P-type ATPase. Similar utilization of either of these compounds was not observed with the preparations of (Na⁺ + K⁺) ATPase. Therefore, differences in the inhibition by ADP-TNP or ATP-TNP between the two enzymes may reflect differences in the stability of the inhibitor and its effective concentration. No such release of inorganic phosphate was observed with either enzyme when AMP-TNP was supplied. Results with AMP-TNP suggest that trinitrophenyl derivatives may be potent inhibitors of the maize root enzyme when derivatives are not degraded, thus making them useful in studies of ATP binding reactions of the enzyme mechanism.

Another ATP derivative tested was AMP-PNP. In this derivative the oxygen between the terminal phosphates has been substituted with N, so that hydrolysis does not occur. The effects of AMP-PNP on ATP hydrolysis by the canine kidney and maize root P-type ATPases were assessed at 2.5 mM Mg²⁺ and 2 mM ATP (Fig. 2). Millimolar concentrations of AMP-PNP were necessary to inhibit ATP hydrolysis by either ATPase. These results contrast with those obtained in our laboratory using the V-type ATPase from maize roots, which is inhibited by micromolar levels of AMP-PNP (Brauer and Tu 1994). The canine kidney enzyme appeared to be slightly more sensitive to inhibition by AMP-PNP at lower concentrations.

Conclusions

Tested inhibitors could be differentiated into three classes based on their inhibitory properties. The first class contained compounds with little or no effect on ATP hydrolysis by either enzyme, and only NBD-Cl was in this

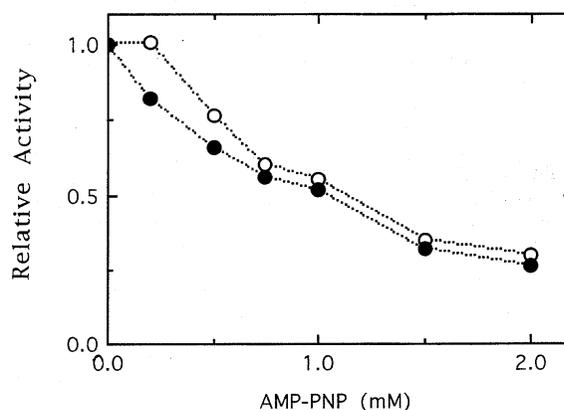


Fig. 2. Effects of AMP-PNP on ATP hydrolysis. Preparations of canine (Na⁺ + K⁺) ATPase (●) and maize root plasma membranes (○) were assayed in the presence of 0 to 2 mM AMP-PNP as described in Materials and methods. Data are plotted relative to the activity in the absence of AMP-PNP, which averaged 1410 ± 30 and 320 ± 11 nmol P_i (mg protein)⁻¹ min⁻¹ for canine and maize root enzyme, respectively.

group. The second class contained compounds that significantly inhibited the (Na⁺ + K⁺) ATPase but had little or no effect on the maize root enzyme and included the reagents FSB \bar{A} , FITC and N₃-ATP. The disparity in the sensitivity of the two enzymes to these three reagents could be an indicator that the ATP hydrolysis pathway of the canine (Na⁺ + K⁺) ATPase differs slightly from that of the maize root enzyme. The third class, which included BzATP and oAMP, contained inhibitors that affected both enzymes to a similar extent. Of these two covalent modifiers, BzATP was superior to oAMP as an inhibitor. Preliminary examination of the characteristics of BzATP inhibition of the maize root H⁺-ATPase supports the hypothesis that BzATP inhibits ATP hydrolysis by covalent modification of amino acids involved in ATP binding or hydrolysis. Research on BzATP as a probe of the ATP hydrolysis pathway of maize root enzyme is continuing.

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