

Nucleotide binding is insufficient to induce cold inactivation of the vacuolar-type ATPase from maize roots

Abstract

Although it has been established that the stability of V-type ATPase at 0 to 4°C is diminished by the presence of substrate, the mechanism of inactivation is poorly understood. The ability of various nucleotide analogs that have been identified as either competitive or non-competitive inhibitors of ATP-dependent proton transport to induce cold inactivation of the maize (*Zea mays* L.) vacuolar-type H⁺-ATPase at 0°C was compared to that of substrate ATP. There was little if any loss in activity over 30 min incubation at 0°C in the presence of 10 mM KNO₃ and 2 mM MgSO₄. Addition of 2 mM ATP promoted significant inhibition over this time period. The noncompetitive inhibitors, 5'-adenylylimidodiphosphate and 2',3'-O-(4-benzoylbenzoyl)-adenosine triphosphate, did not accelerate the loss of activity. Therefore, binding of nucleotides was not sufficient for cold inactivation. The competitive inhibitor, dialdehyde derivative of AMP, did accelerate cold inactivation of the H⁺-ATPase but to a much lesser extent than ATP. During cold incubation, ATP and the dialdehyde derivative of AMP were hydrolyzed, whereas the two non-competitive inhibitors were not. Hydrolysis of nucleotides at 0°C was associated with the degree of cold inactivation. These results suggest that hydrolysis of nucleotides at 0°C rather than binding caused instability in the H⁺-ATPase complex that led to cold inactivation.

Key words

Binding, competitive inhibitor, hydrolysis, non-competitive inhibitor, *Zea mays*.

Abbreviations

AMP-PNP, 5'-adenylylimidodiphosphate; Bz-ATP, 2',3'-O-(4-benzoylbenzoyl)-adenosine triphosphate; oAMP, dialdehyde derivative of AMP; Pi, inorganic phosphate.

INTRODUCTION

There exists at least three categories of transport ATPases, P-, V- and F-type, that link the transport of ions to either the hydrolysis or synthesis of ATP (Nelson and Taiz, 1989). These three types of ATPases can be distinguished by their biochemical, physical and physiological properties. Characteristics of the V-type transport are intermediate between the F- and P-type ATPase. Like the P-type ATPase, the V-type ATPases under normal physiological conditions couples the transport of ions against an electrochemical gradient utilizing the chemical free energy from the hydrolysis of the terminal phosphate from ATP (Nelson and Taiz, 1989; Briskin, 1990). Like the F-type ATPase, a phosphorylated

intermediate is absent with the V-type ATPase and the enzyme is composed of multiple subunits segregated into peripheral and membrane-bound domains (Sze, 1984). In addition, both the V- and F-type ATPase exhibit cold sensitivity which is associated with the release of the peripheral domain from the enzyme complex (Pullman *et al.*, 1960; Griffith *et al.*, 1986; Rea *et al.*, 1987; Arai *et al.*, 1989; Moriyama and Nelson, 1989). In the case of the V-type ATPase, inactivation at 0°C requires the presence of substrate, MgATP, and mild chaotropic salts like either KI or KNO₃ (Griffith *et al.*, 1986; Rea *et al.*, 1987; Arai *et al.*, 1989; Moriyama and Nelson, 1989). Arai *et al.* (1989) hypothesized that binding of ATP to the catalytic site was responsible for cold inactivation since the K_m for ATP hydrolysis was approximately

the same as the ATP concentration that stimulated cold inactivation 50% of the maximum effect. However, the possibility that the hydrolysis of bound ATP induced the instability that leads to cold inactivation was not explored. To address this issue, the degree of cold inactivation in the presence of substrate was compared to that induced by nucleotide like compounds that are either competitive or noncompetitive inhibitors of ATP hydrolysis.

RESULTS

Effects of different nucleotide analogs on cold inactivation

In the presence of 10 mM KNO_3 at 0°C , the addition of 0.5 mM ATP promoted cold inactivation of the corn root V-type ATPase (fig. 1). Over a 90 min period, the vast majority of the proton transport activity was

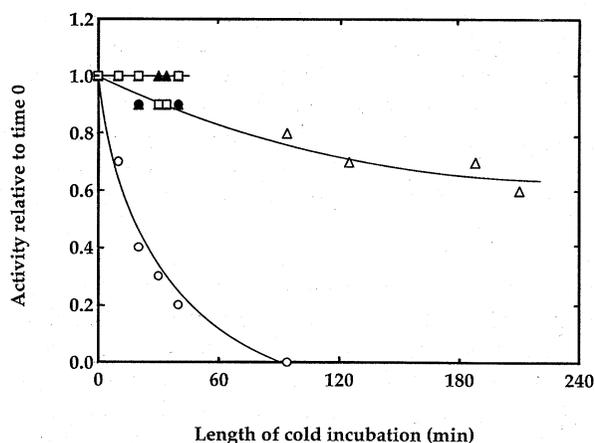


Figure 1. Time course of the loss in proton transport activity during incubation at 0°C . Tonoplast vesicles were incubated with 10 mM KNO_3 and 2.5 mM MgSO_4 in the absence of other additions (□) or the presence of 0.5 mM ATP (○), Bz-ATP (●), AMP-PNP (▲) or oAMP (△) for 0 to 30 min on ice. The activity is plotted relative to the level of proton transport found immediately after the addition of the nucleotide or analog. The activity immediately after additions (0 time) averaged $1.02 \pm 0.04 \text{ A min}^{-1} \text{ mg}^{-1}$ protein in the absence of nucleotide-like compounds, oAMP and ATP, $0.71 \pm 0.03 \text{ A min}^{-1} \text{ mg}^{-1}$ protein in the presence of Bz-ATP and $0.43 \pm 0.02 \text{ A min}^{-1} \text{ mg}^{-1}$ protein in the presence of AMP-PNP. The data are the average of three experiments each with three replicates. Because the coefficient of variation for each mean was less than 5%, the size of data symbols is larger than the mean plus and minus one standard error. The reduction in proton transport activity at 0 time with AMP-PNP and Bz-ATP occurred because dilution of these reagents in the incubation buffer with assay media was not sufficient to overcome inhibition (Brauer and Tu, 1994).

lost in the presence of ATP but not in its absence. The concentration of ATP during cold incubation was approximately the same as the K_m for ATP hydrolysis at this temperature (Tu *et al.*, 1988). The presence of 0.5 mM Bz-ATP or AMP-PNP did not accelerate the loss of proton transport activity during cold inactivation (fig. 1). The concentrations of AMP-PNP and Bz-ATP during cold incubation were 5 to 10 times greater than the K_i values (Brauer and Tu, 1994). Cold inactivation did occur in the presence of oAMP (fig. 1). Similar results were obtained with dialdehyde derivative of ATP (data not shown). The concentration of oAMP during cold incubation was approximately the same as the K_i value (Chow *et al.*, 1992; Brauer and Tu, 1994).

Cold inactivation appeared to follow first-order kinetics (fig. 1). To better quantitative differences in the rate of cold inactivation between various nucleotides, the data in figure 1 were plotted according to first-order kinetics so that half-life for proton transport activity could be determined. In the absence of nucleotides, the half-life for proton transport at 0 to 4°C was $1510 \pm 210 \text{ min}$ ($n=3$). The addition of either Bz-ATP or AMP-PNP did not change this half-life (data not shown). The addition of ATP or oAMP decreased the half-life to $15.7 \pm 0.5 \text{ min}$ ($n=3$) and 306 ± 16 ($n=3$), respectively.

Nucleotide hydrolysis during cold incubation

Experiments were conducted to determine if the degree of nucleotide hydrolysis during cold storage was correlated with the effectiveness of different nucleotide analogs. Of the nucleotides tested, there was only significant hydrolysis of ATP and oAMP during the cold storage period (fig. 2). In the presence of either AMP-PNP or Bz-ATP, less than $0.3 \mu\text{mol}$ of Pi were released after 90 min of cold incubation. Hydrolysis of ATP initially occurred at a rate that was much greater than that observed with oAMP. The rate of Pi formation from either ATP or oAMP could be fitted to first-order kinetics (data not shown). The half-life for Pi formation was similar to those obtained for the loss of proton transport activity, 14.8 ± 0.7 ($n=3$) and $278 \pm 22 \text{ min}$ ($n=3$) in the presence of ATP and oAMP, respectively.

Lack of an effect of ADP on cold inactivation

Two experiments were conducted to test whether the accelerated cold activation in the presence of ATP was due to the presence of ADP. Cold inactivation

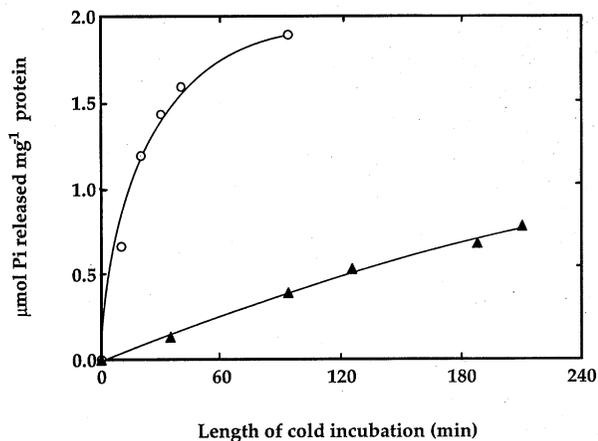


Figure 2. Time course of inorganic phosphate production during cold incubation. Tonoplast vesicles were incubated on ice in the presence of 10 mM KNO₃, 2.5 mM MgSO₄ and 0.5 mM either ATP (○) or oAMP (▲). Aliquots were removed periodically and assayed for inorganic phosphate release as described under Methods. The data are an average of three experiments, each with three replicates. Because coefficients of variation for each mean was less than 5%, the size of the data symbol is larger than the mean plus and minus one standard error.

by ATP was conducted in the presence of pyruvate kinase and phosphoenolpyruvate so that produced ADP could be resynthesized into ATP. Addition of the ATP regenerating system did not alter the degree of cold inactivation (data not shown). Secondly, addition of ADP to the concentrations indicated from ATP hydrolysis data did not accelerate the rate of cold inactivation in the absence or presence of ATP (data not shown). Results from these experiments indicated that the effects of ATP on cold inactivation were due to ATP and not ADP produced from ATP hydrolysis.

DISCUSSION

Cold inactivation in the presence of substrate and mild chaotropic agents is an identifying feature of V-type ATPase from various sources (Griffith *et al.*, 1986; Rea *et al.*, 1987; Arai *et al.*, 1989; Moriyama and Nelson, 1989; Nelson and Taiz, 1989). The existence of at least two types of nucleotide binding sites on the tonoplast enzyme complex has been hypothesized on the basis of sequence analysis (Nelson and Taiz, 1989) and studies with inhibitors (Manolson *et al.*, 1985; Brauer and Tu, 1994). AMP-PNP and Bz-ATP inhibited the tonoplast H⁺-ATPase in a manner that was noncompetitive with respect to ATP (Manolson *et al.*, 1985; Brauer and

Tu, 1994) and did not protect against irreversible, time-dependent inhibition by dialdehyde adenosine nucleotide derivatives (Chow *et al.*, 1992; Brauer and Tu, 1994). These observations formed the basis for hypothesizing the existence of a regulatory nucleotide binding site. Results from this study indicate that nucleotide binding is not sufficient to accelerate cold inactivation. Under conditions in which Bz-ATP and AMP-PNP are inhibitory to proton transport, their presence during cold inactivation did not accelerate inactivation like that observed with the substrate ATP (*fig. 1*). Therefore, binding of nucleotides to the H⁺-ATPase complex did not accelerate cold inactivation.

Both oAMP and ATP accelerated cold inactivation (*fig. 1*). Because oAMP is a competitive inhibitor with respect to ATP (Chow *et al.*, 1992; Brauer and Tu, 1994), it seems likely that both of these compounds bind at or near the same site on the enzyme complex. Therefore, one could conclude that binding of a nucleotide at or near the catalytic site for ATP induces cold inactivation. However, such a conclusion did not adequately account for the difference between these two compounds in their effectiveness to induce cold inactivation. The concentration of both oAMP and ATP during cold incubation in *figure 1* was close to the K_i and K_m values for these two reagents (Brauer and Tu, 1994). Therefore, the bound oAMP and ATP should have been comparable, but ATP was several fold greater in its effectiveness to accelerate cold inactivation.

The formation of Pi from either oAMP or ATP during cold incubation was followed to determine if there was a difference in the rate of hydrolysis of these compounds. The initial rate of oAMP hydrolysis was substantially less than that of ATP (*fig. 2*). In fact, there was a strong association between the half-life of proton transport activity and the half-life of the formation of Pi in the presence of either nucleotide. In addition, when the degree of cold inactivation was plotted as a function of the formation of Pi, the data from both nucleotides were described by the same linear relationship (*fig. 3*). Therefore, there was a strong correlation between enzymatic hydrolysis during cold incubation and the degree of inactivation. Taken together, our data suggest that cold inactivation was associated more with enzymatic turnover of nucleotides rather than binding to the complex. The catalytic turnover of ATP may induce a loosening of the association between the peripheral and integral domains of the ATPase complex, which leads to dissociation and

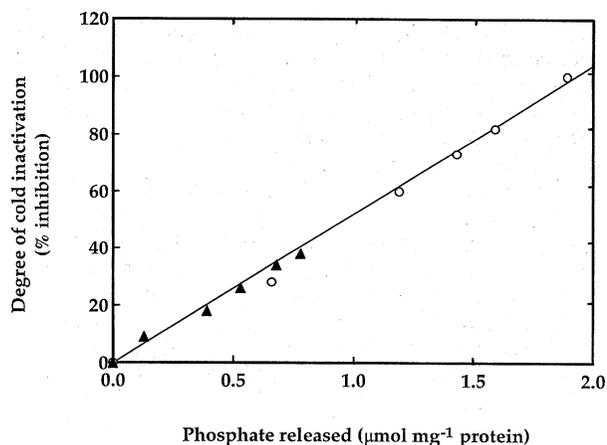


Figure 3. Association between inorganic phosphate formation and degree of cold inactivation in the presence of either oAMP (▲) or ATP (○). Proton transport and phosphate formation data from figures 1 and 2, respectively, were correlated. The line in the figure represents the regression equation generated from the data, which had a correlation coefficient of 0.99. The coefficient of variation for each mean was less than 5%.

inactivation. Because inactivation occurs more readily at lower temperatures (Rea *et al.*, 1987; Moriyama and Nelson, 1989), the association between enzyme domains probably involves hydrophobic interaction rather than electrostatic forces. The intensity of the hydrophobic interaction that holds the enzyme domains together probably changes in response to conformational transitions in protein structure due to nucleotide hydrolysis. In addition, the results of this study supports our earlier contention that there are at least two different types of nucleotide binding sites on the H⁺-ATPase complex. One of these sites at which ATP is hydrolyzed is also involved in the mechanism that is responsible for cold inactivation. The other site(s) at which AMP-PNP and Bz-ATP interact with the enzyme is not involved in cold inactivation mechanism.

METHODS

Tonoplast isolation. Roots from three day-old seedlings of corn (*Zea mays* L. CV FRB 73) were grown and harvested as described previously (Nagahashi and Baker, 1984). Highly purified tonoplast vesicles were prepared by differential centrifugation followed by isopycnic sucrose gradient centrifugation (Tu *et al.*, 1987). These vesicles are essentially devoid of markers for endoplasmic reticulum, Golgi, plasma membranes and mitochondria (Tu *et al.*, 1987). Protein concentration was determined by a modification

of the Lowry procedure (Bensadoun and Weinstein, 1976). Modification of the tonoplast H⁺-ATPase by oAMP was performed essentially as described by Chow *et al.* (1992).

Enzyme assays. Proton transport was assaying by following changes in the absorbance of acridine orange at 492 nm as described previously (Tu *et al.*, 1987). No proton transport activity was observed in the presence of 50 mM KNO₃ (Tu *et al.*, 1987) indicating the vast majority of the activity was catalyzed by a V-type ATPase. The specific activity of the H⁺-ATPase in maize root tonoplast vesicles averaged 1.0 ± 0.04 A min⁻¹ mg⁻¹ protein in the presence of 2 mM Mg-ATP and 50 mM KCl throughout these studies. The hydrolysis of ATP and other phosphate containing compounds was followed by the appearance of phosphate as determined colorimetrically as molybdate-malachite green complex (Tu *et al.*, 1987).

Cold inactivation. Tonoplast vesicles were combined with various compounds as indicated elsewhere and held on ice. After incubation for 0 to 30 min, 100 µl aliquots were removed and diluted to 2.0 ml of proton transport assay medium. After 5 min at 18 to 22°C, proton transport was determined as described above. The activity remaining after incubation (5 to 30 min) as compared to the activity immediately after the addition of compounds (zero time) was used as a measure of cold inactivation (Moriyama and Nelson, 1989).

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