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ATP:AMP phosphotransferase activity, a new characteristic of *Catharanthus roseus* tonoplasts

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The ATPase activity of *Catharanthus roseus* tonoplasts was examined using HPLC separation and quantification of adenine nucleotides. ATP seemed to be degraded into ADP and AMP by tonoplast vesicles. When ADP was the initial substrate, the appearance of AMP and concomitant ATP synthesis were observed; these reactions were inhibited by Ap^5A. The apparent degradation of ATP into AMP was also inhibited by Ap^5A. These results indicated that AMP arose from an ATP:AMP phosphotransferase activity and excluded the possibility of the hydrolysis of ADP into AMP by the tonoplast ATPase. AMP was degraded by the microsomal fraction from protoplasts or by the cytosol while the tonoplast vesicles did not hydrolyze it. This observation was used to assess the purity of tonoplasts.

ATP:AMP phosphotransferase; Enzyme inhibition; P^1,P^5-Di(adenosine-5') pentaphosphate; Tonoplast; ATPase; (*Catharanthus roseus*)

1. INTRODUCTION

The tonoplast Mg^{2+}-ATPase is certainly the most extensively studied activity among the H^+-pumping enzymes linked to the vacuolar membrane [1–4]. At present, the tonoplast ATPase is considered as a marker for the vacuolar membrane [5]. However, its differentiation from other ATPases, originating from the plasmalemma, mitochondria, Golgi apparatus, etc. is complex; indeed, this tonoplast ATPase is mainly characterized through a very variable degree of nitrate inhibition [6–10].

To study ATPase activities, we have developed an HPLC method for the simultaneous measurement of adenine nucleotides [11].

In the presence of 1 mM ammonium molybdate, described as a phosphatase inhibitor [12], when ATP was the initial substrate, two degradation products, ADP and AMP, were detected. With ADP as initial substrate, AMP appeared and ATP synthesis was observed.

In order to discriminate between a nucleotidase activity or an ATP:AMP phosphotransferase activity, kinetic studies were performed in the presence of Ap^5A, a potent inhibitor of adenylate kinase [13,14]. An ATP:AMP phosphotransferase activity was revealed.

New experimental conditions were defined for studying the ATPase activity and its inhibition by nitrate.

To assess the purity of tonoplast vesicle preparations, a simple HPLC test control is suggested.

2. MATERIALS AND METHODS

ATP, ADP, AMP and Ap^5A were purchased from Sigma. All other chemicals were purchased from Merck.

The separation of nucleotides was achieved on a Supelcosil LC-18-DB (5 μm, 15 cm × 4.6 mm i.d.) column protected by a Brownlee (7 μm, 1.5 cm × 3.2 mm i.d.) guard column as described previously [11]. Briefly, HPLC was carried out with a Gilson autoanalytic gradient system and nucleotides were detected at 259 nm using a Beckman model 165 UV spec-

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Abbreviations: Ap^5A, P^1,P^5-di(adenosine-5') pentaphosphate; HPLC, high-performance liquid chromatography

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trophotometer. All operations, sample injections, isocratic elutions, UV detections, peak integrations and column regenerations, were automatically controlled by means of an HPLC system manager software; the system operated under full automation from sample injection to data report.

Protoplasts, vacuoles and tonoplast vesicles were prepared as in [15,16]. Protein concentrations were measured by the method of Read and Northcote [17]. Kinetic studies were carried out essentially as in [11].

3. RESULTS AND DISCUSSION

Current data on the phosphohydrolase activities of vacuoles or tonoplasts describe the hydrolysis of ATP, ADP and AMP in the presence [12,18–21] or absence [22,23] of molybdate. These data are usually obtained from P_i titrations and obviously cannot account for tonoplast ATPase activity when ATP is the substrate and P_i the titrated product. We have demonstrated previously that HPLC is a suitable technique for obtaining balances of complex hydrolysis of adenine nucleotides [11,16].

This technique was applied to the 5′-nucleotidase activities of the tonoplast (prepared from purified vacuoles) in the systematic presence of 1 mM molybdate.

No degradation of AMP was detected in the presence of tonoplast vesicles while it was hydrolyzed by the cytosol and the microsomal fraction isolated from protoplasts (fig.1). Thus, these last fractions contained enzymatic activities which degraded AMP and, among the degradation products, adenosine and adenine were unambiguously identified during investigation of the kinetics (not shown). With tonoplast vesicles, less than 3% contamination by the microsomal fraction from protoplasts could be detected using the AMP assay. On the basis of these observations, the AMP assay, associated with the usual enzymatic markers, gave reliable data on the eventual contaminations of tonoplast vesicles.

Two adenine nucleotides, ADP and AMP, arose from ATP degradation by tonoplast vesicles of *Catharanthus roseus* [16] and *Acer pseudoplatanus* [11]. The appearance of AMP could reveal either an ADPase, a phosphotransferase, an organic pyrophosphatase or a non-specific ATPase activity. In view of our previous results [11,16], the kinetics were analyzed in the presence of 1 mM Ap^5A, a potent inhibitor of adenylate kinase.

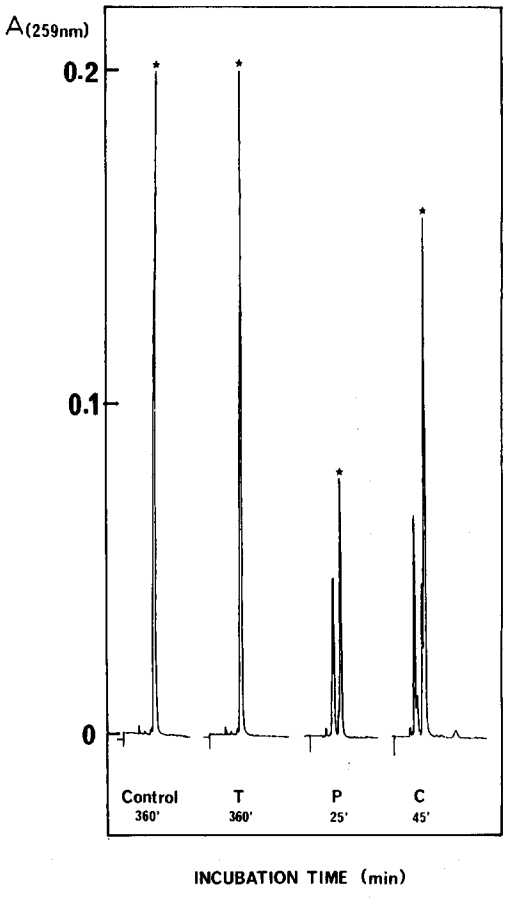


Fig.1. Sample chromatograms illustrating AMP (*) hydrolysis in the presence of cytosol (C), microsomal fraction isolated from protoplasts (P) or tonoplast (T) from *C. roseus*. Initial substrate, 3 mM AMP. Incubation medium: 0.3 ml of 50 mM Tris-Mes buffer at pH 7 containing 5 mM $MgCl_2$, 50 mM KCl, 1 mM molybdate and C (500 μg) or P (1 mg) or T (16 μg), respectively. Chromatographic conditions: Supelcosil LC-18-DB (5 μm, 15 cm × 4.6 mm i.d.) column. Solvent system: 100 mM KH_2PO_4, 25 mM tetra-*n*-butylammonium hydrogen sulfate. pH 7, 18% methanol (v/v); isocratic HPLC elution; flow rate, 2 ml/min.

Under these conditions, the AMP level decreased to 25% (fig.2) without markedly affecting the ATPase activity (not shown).

We have already shown that ADP was converted into AMP and that concomitant ATP synthesis occurred [11,16]. ATP synthesis by tonoplast vesicles was 87% inhibited by 1 mM Ap^5A (fig.3A), but the appearance of AMP was not inhibited to such an extent (75%) (fig.3B).

From these observations, it is clear that the ma-

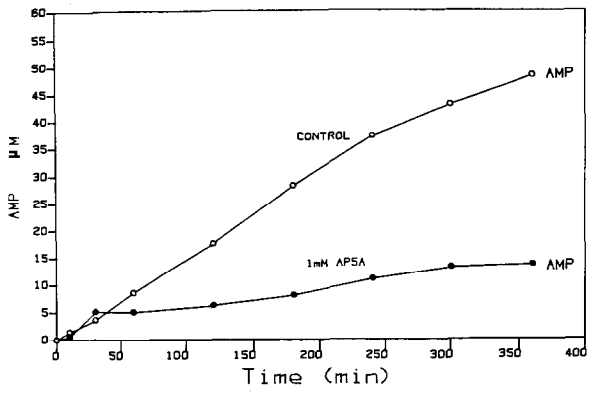


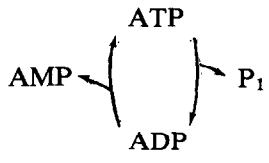
Fig.2. Inhibition of AMP formation by 1 mM Ap^5A. Initial substrate, 3 mM ATP. Incubation medium, as in fig.1, contained 16 μg tonoplast from *C. roseus*, with (●) or without (○) 1 mM Ap^5A.

jor part of AMP results from an ATP:AMP phosphostransferase activity according to the reaction: $2ADP \rightleftharpoons ATP + AMP$.

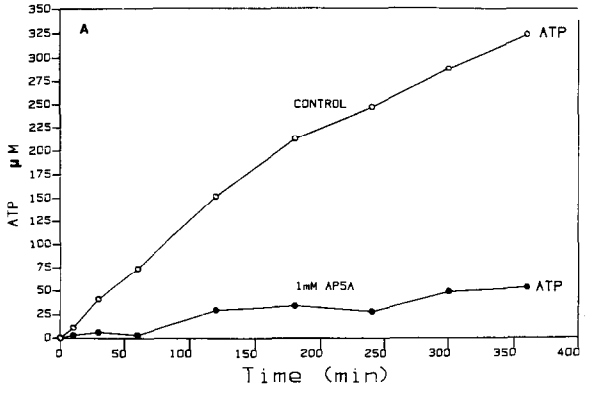
Since the appearance of AMP and the synthesis of ATP were not inhibited to the same extent by Ap^5A, a residual ADPase activity cannot be excluded [24]. Further investigations are required to demonstrate whether this residual activity is due to the existence of a tonoplast ADPase.

Enzymes which hydrolyze Ap^5A have been described [25,26]. Under our experimental conditions, no hydrolysis of Ap^5A by tonoplast vesicles was detected on HPLC.

To our knowledge, no report has been published on a tonoplast phosphotransferase activity. This activity may be an important component of the so-called 'energy charge' concept and may maintain equilibrium between the adenine nucleotides. This phosphotransferase activity may be involved in a regulation process of adenine nucleotides according to the following scheme.



Thom and Komor [20] have pointed out that the total P_i released during ATP hydrolysis was 30%



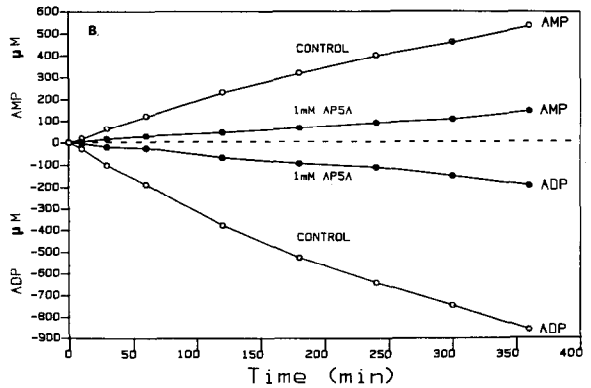


Fig.3. (A) Inhibition of ATP synthesis by 1 mM Ap^5A; initial substrate, 3 mM ADP. (B) Inhibition of ADP and AMP evolution by 1 mM Ap^5A; initial substrate, 3 mM ADP. Experimental conditions as in fig.1 except that the incubation medium contained 11 μg tonoplast from *C. roseus*, with (●) or without (○) 1 mM Ap^5A.

higher than the γ-phosphate arising from [γ-^{32}P]ATP hydrolysis, the explanation proposed being the hydrolysis of ADP by tonoplast ATPase. We suggest another hypothesis to account for the lack of correlation between total P_i liberated and amount of γ-^{32}P: ATP is partly regenerated by the phosphotransferase and the ATPase is supplied by this reaction.

Accordingly, the major part of P_i released from ADP either by an ATPase [12,19–22] or by an ADP-dependent proton pump [24] may be interpreted according to our hypothesis.

As shown by our results, the tonoplast ATPase activity must be measured in the presence of Ap^5A to avoid ATP regeneration. Under these conditions, the ATPase activity was inhibited 40% by 50 mM potassium nitrate (fig.4).

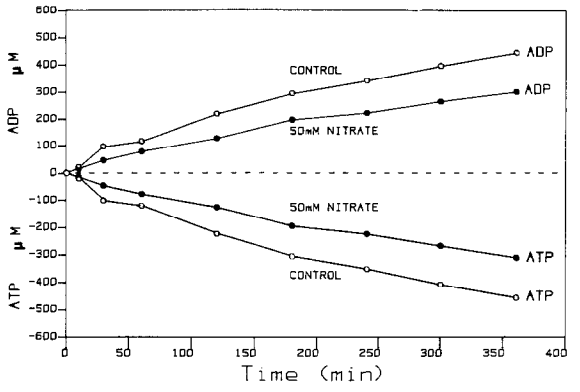


Fig.4. Inhibition of ATP hydrolysis by NO_3^-. Initial substrate 3 mM ATP in the presence of 1 mM Ap^5A. Incubation medium, as in fig.1, contained 16 μg tonoplast from *C. roseus,* with (●) or without (○) 50 mM KNO_3.

In conclusion, new features have been found in addition to the known properties of tonoplasts: (i) the absence of enzymatic activities involving AMP degradation in contrast with the cytosol and the microsomal fraction of protoplasts; (ii) the presence of a tonoplast-bound ATP:AMP phosphotransferase.

This new characteristic activity, demonstrated on *A. pseudoplatanus* and *C. roseus,* will be useful in understanding the mechanism of adenine nucleotide interconversion in tonoplasts.

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