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THE EFFECT OF BIVALENT METAL IONS ON ATPase–ADPase ACTIVITIES OF APYRASE FROM *SOLANUM TUBEROSUM*

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Key Word Index—*Solanum tuberosum*; Solanaceae; potato; apyrase pyrophosphohydrolase; ATPase-ADPase.

Abstract—The purpose of this study was the elucidation of the possible importance of bivalent metal ions in controlling the activity of apyrase (ATP: diphosphohydrolase EC 3.6.1.5) purified from tubers of *Solanum tuberosum* cv Desirée. Similarities between the K_m and V_m values for ADP and Ca^{2+} suggest that the true substrate of this enzyme is the metal ion-nucleotide complex. The association constant of the Ca-ADP complex was measured under the same conditions of pH and ionic strength as in the enzymatic assay system in order to calculate the true concentration of this complex. In contrast, $[Mn(H_2O)_6]^{2+}$ spin resonance spectroscopy (ESR) showed that apyrase binds this paramagnetic metal ion in the absence of ATP or ADP. The spectrum of $[Mn(H_2O)_6]^{2+}$ showed a transition at low field after the addition of apyrase. This result indicates that the binding of the enzyme produces a distortion in the electronic symmetry of $[Mn(H_2O)_6]^{2+}$. Apyrase binds other bivalent cations because hysteretic behaviour is observed when the enzyme is preincubated with bivalent metal ions in the absence of nucleotide; this hysteretic behaviour can be interpreted as a displacement of a ligand strongly bound to the enzyme (M^{2+}) by the substrate (Ca-ATP or Ca-ADP). The contents *in vivo* of Ca, Mg, Mn, Co, Zn, ATP and ADP were determined.

INTRODUCTION

Plant ATPases have been classified by Serrano [1] as plasma membrane ATPases, mitochondrial ATPases, vacuolar ATPases and apyrases (soluble) according to the following characteristics: optimum pH, substrate specificity, effects of different type of inhibitors on enzymatic activity, bivalent metal ion requirements and subcellular localization.

For some time our group has been interested in apyrase (ATP: diphosphohydrolase EC 3.6.1.5) from potato tuber [2–4]. This enzyme hydrolyses ATP and ADP finally producing AMP and inorganic phosphate. Although these are probably the substrates *in vivo*, apyrase has a rather low specificity for the non-phosphate part of the molecule. On the other hand, changes in the pyrophosphate chain produce significant effects on the catalytic but not the binding properties of the enzyme [5, 6].

We have also reported that tyrosyl, tryptophyl, arginyl and dicarboxylic groups are the possible amino acid residues participating in the catalytic process [6, 7]. Finally, from the determination of some physicochemical and kinetic characteristics, we have found distinct isoenzymes present in the different clonal varieties of *S. tuberosum* [8, 9].

Abbreviations: ESR, electron spin resonance; BSA, bovine serum albumin; AMP-PCP, 5′-(β, γ-methylene) triphosphate; MES, 2-*N*-morpholinoethanesulphonic acid; TES, *N*-tris-(hydroxymethyl-2-aminoethanesulphonic acid).

Apyrases belong to that group of enzymes which are activated by metal cations; they are inactive in the absence of bivalent metal ion [10]. In a previous report we compared the stimulatory effect of Ca^{2+} with that of other bivalent metal ions [6]. The most relevant finding was that some metal ions, especially Mn^{2+} and Cd^{2+}, modify the ATPase/ADPase activity ratio. No correlation was found between these kinetic differences and the ionic radius, the metal ion-nucleotide association constant or the electronegativity of the bivalent cation.

The purpose of this study was the further investigation of the possible role of bivalent metal ions in the hydrolytic reaction catalysed by apyrase from potato tuber, isolated from cv Desirée. This involved a study of enzyme-metal ion-substrate coordination through kinetic and binding studies.

RESULTS AND DISCUSSION

K_m *and* V_m *for* Ca^{2+} *and ADP*

Table 1 summarizes the kinetic parameters for Ca^{2+} with ADP as fixed substrate, and for ADP with $[Ca^{2+}]$ constant. The results show a similarity between K_m and V_m values for ADP and Ca^{2+}, suggesting that the 'active' form of the nucleotide is the metal ion-nucleotide complex with the usual 1:1 stoichiometry [11]. The linearity of plots of S/v and $[M^{2+}]/v$ against [ADP] or $[Ca^{2+}]$ rule out an inhibitory effect of free nucleotide or Ca^{2+}.

The association constant of the Ca-ADP complex was measured at the same pH and ionic strength as obtained

Table 1. Kinetic parameters of apyrase

		K_m ($\times 10^3$ M^{-1})	V_m ($\times 10^3$ M^{-1} min)
ADP	Ca^{2+}(2 mM)	0.065	546
Ca^{2+}	ADP(2 mM)	0.062	522
Ca-ADP*	—	0.037†	534

*Complex concentrations were calculated from an association constant equal to 638 M^{-1} calculated at pH 6.0.

†These values were calculated from the Hanes–Woolf plot of the true complex concentrations and the mean rates obtained from experiments with ADP and Ca^{2+} shown above.

in the enzymatic assay in order to calculate its true concentration in the medium. Two different methods were employed: gel filtration through Sephadex G-10 and direct titration with a Ca^{2+}-selective electrode. The mean values obtained were 643 ± 95 M^{-1} ($n=6$) and 633 ± 73 M^{-1} ($n=4$, from independent titration curves) respectively. An average value of 638 M^{-1} was used for the calculation of the complex. Table 1 shows the K_m value assuming Ca-ADP to be the active substrate. Although the same parameters were not calculated for ATP and Ca^{2+}, it is reasonable to suppose that the hydrolysis of both nucleotides proceeds through the same mechanism.

In a previous paper we reported that Cr (III)-ATP was a competitive inhibitor of the enzyme [6]. Because of the tightness of binding of the metal ion in this substrate analogue, it can be proposed that the metal ion-nucleotide complex interacts with the enzyme through the pyrophosphate chain and not through the metal ion [12, 13]. The observation that other bivalent ions, in addition to Ca^{2+}, produce stimulatory effect on apyrase [6] support the proposal that the metal ion is not acting as a bridge between the enzyme and the substrate. It has generally been observed that Ca^{2+} has an inhibitory effect on metal ion bridge enzymes, but activates those with substrate bridge complexes [11].

The ESR spectrum of Mn-*apyrase*

The spectrum of $[Mn(H_2O)_6]^{2+}$ changed after the addition of apyrase, showing a transition at low field and a decrease in the height of the six lines typical of the Mn^{2+} (Fig. 1). This indicates that the enzyme binds to Mn^{2+} in the absence of substrate and that this binding produces a distortion in the electronic symmetry of $[Mn(H_2O)_6]^{2+}$. The altered spectrum can be interpreted as a distortion in the inner coordination sphere of $[Mn(H_2O)_6]^{2+}$ bound to apyrase [14]. There are several known examples of enzymes where large deviations of the cubic symmetry of $[Mn(H_2O)_6]^{2+}$ are seen in further transitions of the isotropic region of the spectrum with the consequent decrease of the height of the six lines [14–18].

It was not possible directly to measure the stoichiometry of this binary complex. However, the value for the dissociation constant, as calculated from the decrease in line height, was *ca* 40 μM.

No further changes in the ESR spectrum of the Mn^{2+}-apyrase binary complex were observed when 5 mM $CaCl_2$ was added before or after the paramagnetic cation. These results would suggest that Ca^{2+} does not interact with apyrase under these conditions, that both ions have

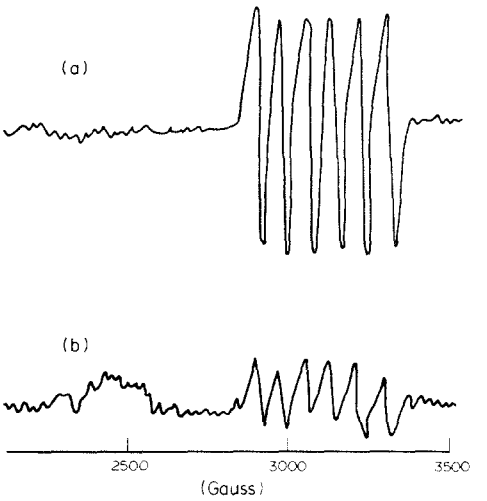

Fig. 1. ESR spectrum for $[Mn(H_2O)_6]^{2+}$ solution. The spectrum was run in 0.1 M TES pH 8.0 at $27 \pm 1°$ with an amplitude modulation of 80 and a signal level of 3200. (a) The solution contained 0.5 mM $[Mn(H_2O)_6]^{2+}$. (b) The solution contained 0.49 mM $[Mn(H_2O)_6]^{2+}$ and 0.46 mM apyrase.

different binding sites, or if both cations bind to the same site, Mn^{2+} has a higher affinity for apyrase than Ca^{2+}. This point will be discussed further in the next section. The addition of EDTA led to the disappearance of both typical sextet of $[Mn(H_2O)_6]^{2+}$ and the low field transition.

Hysteretic behaviour of metal ion-apyrase binary complexes

Apyrase binds other bivalent metal ions besides Mn^{2+}, evidenced by the appearance of hysteretic behaviour when the enzyme was preincubated with bivalent cations in the absence of a nucleotide substrate. The kinetics of hydrolysis of Ca-ATP and Ca-ADP catalysed by apyrase preincubated with 0.25 mM Mn^{2+} are shown in Fig. 2. Controls contained either sodium chloride alone or 10 mM EDTA in addition to the bivalent cation. The lag period was also observed with Ca^{2+}, Mg^{2+}, Co^{2+} and Zn^{2+}; the lag periods extrapolated from the respective kinetic curves are given in Table 2. Mn^{2+} and Cd^{2+} produced the largest effects consistent with the proposal that these ions bind most strongly to the enzyme. A similar lag period in the kinetic of hydrolysis is yielded by preincubation with Ca^{2+} in the pH range 4.0–8.0. The larger lag period produced by Mn^{2+} support the ESR experiments in which no displacement of Mn^{2+} by Ca^{2+} was observed at pH 8.0.

According to Frieden [19], this hysteretic behaviour may be due to: (i) molecular aggregation of the enzyme, (ii) displacement of a ligand strongly bound to the enzyme or (iii) isomerization of enzymatic forms. The hysteresis due to molecular aggregation was discounted because the molecular mass of apyrase was unchanged during the course of the experiments. The molecular mass of Ca^{2+}-apyrase determined by gel filtration through Sephadex G-100 under hysteretic conditions (Ca^{2+}, pH, temperature) was 47 500. This value is

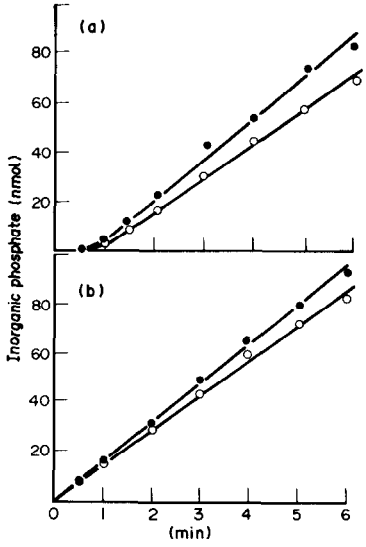

Fig. 2. The kinetics of hydrolysis of ATP and ADP catalysed by Desirée apyrase preincubated with metal ions. (a) Preincubation with 0.25 mM $MnSO_4$. (b) Preincubation with 0.75 mM NaCl or 0.25 mM $CaCl_2$ and 10 mM-EDTA. After one hr of preincubation of the enzyme at 4° in 0.1 M MES, pH 6.0 a sample of the mixture was diluted in BSA (0.2 mg/ml) and the release of inorganic phosphate from ATP and ADP was measured. The assay medium contained 0.05 mM nucleotide; 5 mM $CaCl_2$; 0.1 M MES, pH 6.0.

Table 2. Lag period of apyrase preincubated with bivalent cations

M^{2+} preincubation (Final concentrations 0.25 mM)	Lag period* ATPase (s)	ADPase (s)
Ca^{2+}	18	12
Mg^{2+}	21	30
Mn^{2+}	42	42
Co^{2+}	51	21
Zn^{2+}	15	9

*Lag period extrapolated to inorganic phosphate = 0 (19). Preincubation was in 0.1 M MES, pH 6.0, at 4°. After dilution in BSA (0.2 mg/ml) inorganic phosphate production from Ca-ATP and Ca-ADP was measured as described in the Experimental section. The assay medium contained 0.05 mM nucleotide, 5 mM $CaCl_2$; 0.1 M MES, pH 6.0.

similar to that previously described for apyrase (49 000) in the absence of Ca^{2+} [6].

On the other hand, the displacement of a ligand strongly bound to the enzyme is supported by experimental data because increasing substrate concentration (Ca-ATP or Ca-ADP) produce a decrease in the lag period until it is no longer observed (Fig. 3). In addition, ATP analogues (such as AMP-PCP and Cr(III)-ATP) prevent the hysteretic behaviour of the enzyme. The hysteretic kinetics of apyrase at low concentration of substrates is reversed by EDTA as shown in Fig. 2 (control experiment). The interactions between apyrase and these bivalent cations were however, abolished neither by dilution in BSA (0.2 mg/ml) nor by dialysis against 0.1 M NaCl (Chelex treated).

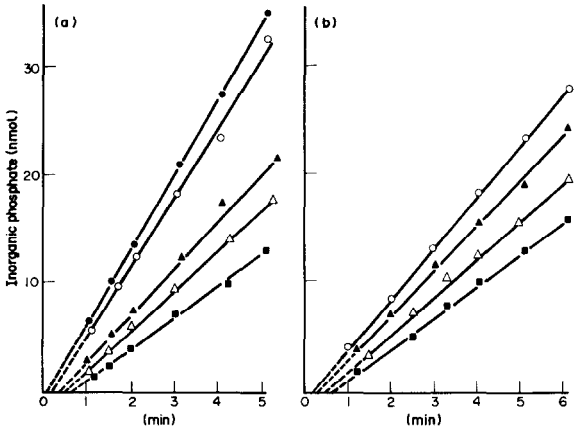

Fig. 3. Effect of metal ion-substrate concentration on the lag period of Ca^{2+} preincubated Desirée apyrase. Preincubation conditions were: one hr at 4° in 100 mM MES pH 6.0; 0.25 mM Ca^{2+}. Assay conditions: 100 mM MES pH 6; 5 mM $CaCl_2$ and variable concentrations of nucleotide: ■——■ 25 μM, △——△ 33 μM, ▲——▲ 50 μM, ○——○ 67 μM, ●——● 100 μM. (a) ATP ranged from 25 to 100 μM. (b) ADP ranged from 25 to 67 μM.

Hatfield *et al.* [20] described some kinetic models to explain the lag period produced by displacement of a strongly bound ligand to the enzyme. One of these fitted our observations of the decrease in the lag period with increasing concentrations of substrate (Ca-ATP or Ca-ADP). According to this model, there is a linear dependence between the apparent rate constant for the approach to steady state (the exponential part of the progress curve) and the substrate concentration (data not shown).

The observation that the bivalent cation-apyrase binary complex does not show hydrolytic activity, discounts the presence of a dual metal ion coordination complex as found with other ATP-dependent enzymes [21]. In these cases one cation is bound directly to the enzyme, the second being coordinated to the nucleotide.

The formation of a M^{2+}-apyrase binary complex may conceivably have a regulatory significance *in vivo*. This non-active enzymatic form may reversibly activated by the true substrate (Ca-ATP or Ca-ADP) by displacement of the bound metal ion. We have therefore, determined the contents of nucleotides and some bivalent metals in potato tuber tissue.

ATP, ADP and some bivalent cation contents in potato tuber

The total content of Ca, Mg, Mn, Zn, ATP and ADP in Desirée potato tubers are shown in Table 3. The percentage of water in different tubers was 77.9% ± 0.9 ($n = 6$); this constancy allowed the expression of the results in terms of fresh weight. The metal contents found are similar to those reported by others for other varieties of potato [22], with the exception of Zn and Co. Comparison between the contents of ATP and ADP and those of the metals suggest that the concentrations of Ca^{2+} and

Table 3. Content of some metal ions and adenine nucleotide of *S. tuberosum* tuber cv Desirée

	Content (μmol/g fr. wt)
ATP	0.148 ± 0.011 (3)
ADP	0.103 ± 0.008 (3)
Mg	4.49 ± 0.56 (6)
Ca	2.70 ± 0.052 (6)
Zn	0.047 ± 0.0058 (6)
Mn	0.026 ± 0.0047 (6)
Co	0.004 ± 0.0002 (6)

Samples were prepared and assayed as described in the Experimental section. Results are means ± s.d. with the number of independent observations shown in parentheses.

Mg^{2+} are at least one order of magnitude greater than those of the nucleotides *in vivo*. Whether or not this obtains *in vivo* depends on the proportion of the total metal in the ionic form and the intracellular and extracellular locations of the various reactive species.

These results suggest to us that Mg^{2+} and Ca^{2+} should be considered as the physiological cations, producing the active form of the substrate (metal ion-nucleotide complex) or/and regulating apyrase activity. Mn^{2+} cannot be entirely dismissed, however, for its strong binding to apyrase might outweigh the fact that the metal ion concentration may well be one and two orders of magnitude lower than those of Ca^{2+} and Mg^{2+} respectively. It is possible that changes in the concentrations of some metal ions as well as in the concentrations of ATP and ADP in the potato tuber tissue might be involved in the regulation of apyrase. In our proposed model, the metal ions which bind to apyrase in the absence of substrate are inhibitors and the metal-nucleotide complex is a activator. This complex would displace the bound metal producing the reactivation of the apyrase (accounting for the hysteretic behaviour).

EXPERIMENTAL

Material. All chemicals were reagent grade. ATP, ADP, TES, MES, PEP, NADP, NADH, hexokinase type F-300, glucose-6-phosphate dehydrogenase type XV, pyruvate kinase type III and lactate dehydrogenase type XI were bought from Sigma. Apyrase was obtained from a pure strain of *Solanum tuberosum* cv Desirée supplied by the Instituto de Investigaciones Agropecuarias 'La Platina', Santiago, Chile. Desirée apyrase was purified as previously described in ref. [6].

Methods

Assay of apyrase. Apyrase activity was assayed by measuring the liberation of inorganic phosphate [23] from ATP and ADP [2]. Variations in the composition of the incubation mixture are given in the legends of Tables and Figures.

K_m and V_m for Ca^{2+} and ADP. The medium contained 0.1 M MES; 0.065 mM EDTA, pH 6.0, throughout. K_m and V_m values were calculated from Hanes–Woolf plots [24] by the method of least squares.

Determination of the association constant of Ca-*ADP.* This was determined by two different methods. (i) A solution containing 2 mM ADP in 0.1 M MES pH 6.0 (Chelex-100 treated) was titrated with a Ca^{2+} sensitive electrode (F-2112 Ca, Radiometer) previously calibrated in the presence of the same buffer solution. (ii) Gel filtration with Sephadex G-10 as described by Hummel and Dreyer [25] was also used. The column was equilibrated with 0.1 M MES pH 6.0. The Ca^{2+} concentration was 0.16 mM. The experiment was initiated by addition of 5 μmol of ADP in 0.1 ml of the same buffer. The total nucleotide concentration was followed by absorbance at 258 nm and the total calcium was measured by atomic absorption spectrophotometry.

The ESR spectrum of Mn^{2+}-apyrase. The ESR spectra were obtained with a Varian Spectrometer model V-4502 operating at 9.1 GHz at 27 ± 1°. The spectra of 0.5 mM Mn^{2+} and 0.5 mM Mn^{2+}-apyrase in 0.1 M TES buffer, pH 8.0 were measured. TES buffer was previously freed from bivalent metal ions with Chelex X-100. The enzyme was treated with 0.1 mM EDTA. The EDTA was later removed by ultrafiltration with 0.1 M TES, pH 8.0.

ATP, ADP and metal contents of the potato tuber. 5 g of peeled tuber was thinly sliced and homogenized in 2.5 M $HClO_4$ in a Sorvall-Omnimixer for 2 min at 0°. After centrifugation (27 000 *g*) for 50 min at 4° each sample was extracted again with $HClO_4$. Both supernatants were brought to pH 7.0 with 10 M KOH and the whole was finally centrifuged at 4° to remove $KClO_4$. Each sample was then freeze-dried and redissolved in 5 ml of dist. H_2O. ATP and ADP concentration were measured enzymaticaly [26, 27]. Metal contents were measured, in sample of ash, from potato tubers, by neutron activation analysis.

Kinetics of apyrase preincubated with bivalent metals. Apyrase (0.08 mM) was incubated at 4° for 1 hr, with 0.1 M MES, pH 6.0 and 0.25 mM-[metal ion] (Ca^{2+}, Mg^{2+}, Mn^{2+}, Co^{2+} or Zn^{2+}). In control experiments, preincubations were done with 0.75 mM NaCl instead of the bivalent cation or by addition of 10 mM EDTA as well as Ca^{2+}. The kinetics of each preincubation were followed after dilution with BSA (0.2 mg/ml). ATPase and ADPase activity of apyrase solutions were determined in 0.1 M MES; 5 mM $CaCl_2$, and 0.025–0.1 mM[ATP] or [ADP], pH 6.0.

Molecular mass determination of Ca-*apyrase.* Molecular mass was calculated from a calibrated Sephadex G-100 column equilibrated with 0.1 M MES and 0.25 mM $CaCl_2$, pH 6.0 [28].

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