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Potato tuber isoapyrases: Substrate specificity, affinity labeling, and proteolytic susceptibility

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Abstract

Apyrase/ATP-diphosphohydrolase hydrolyzes di- and triphosphorylated nucleosides in the presence of a bivalent ion with sequential release of orthophosphate. We performed studies of substrate specificity on homogeneous isoapyrases from two potato tuber clonal varieties: Desirée (low ATPase/ADPase ratio) and Pimpernel (high ATPase/ADPase ratio) by measuring the kinetic parameters K_m and k_{cat} on deoxyribonucleotides and fluorescent analogues of ATP and ADP. Both isoapyrases showed a broad specificity towards dATP, dGTP, dTTP, dCTP, thio-dATP, fluorescent nucleotides (MANT-; TNP-; ethene-derivatives of ATP and ADP). The hydrolytic activity on the triphosphorylated compounds was always higher for the Pimpernel apyrase. Modifications either on the base or the ribose moieties did not increase K_m values, suggesting that the introduction of large groups (MANT- and TNP-) in the ribose does not produce steric hindrance on substrate binding. However, the presence of these bulky groups caused, in general, a reduction in k_{cat}, indicating an important effect on the catalytic step. Substantial differences were observed between potato apyrases and enzymes from various animal tissues, concerning affinity labeling with azido-nucleotides and FSBA (5′-*p*-fluorosulfonylbenzoyl adenosine). PLP-nucleotide derivatives were unable to produce inactivation of potato apyrase. The lack of sensitivity of both potato enzymes towards these nucleotide analogues rules out the proximity or adequate orientation of sulfhydryl, hydroxyl or amino-groups to the modifying groups. Both apyrases were different in the proteolytic susceptibility towards trypsin, chymotrypsin and Glu-C.

Keywords: *Solanum tuberosum*; Solanaceae; Potato tuber; ATP diphosphohydrolase; Desirée and Pimpernel isoapyrases; Substrate specificity; Proteolysis

1. Introduction

ATP-diphosphohydrolase (apyrase, E-type ATPase, ATPDase, NTDase EC 3.6.1.5) hydrolyzes pyrophosphoric bonds of organic and inorganic compounds in the presence of a bivalent metal ion. This activity has been established both in plant and animal tissues. In animal tissues, it has been found as a plasma membrane-bound protein (Valenzuela et al., 1989; Komoszynski, 1996; Wang and Guidotti, 1998; Bigonnesse et al., 2004), and also with an intracellular location associated

Abbreviations: ε-ATP, 1,N^6-ethenoadenosine triphosphate; ε-ADP, 1,N^6-ethenoadenosine diphosphate; FSBA, 5′-*p*-fluorosulfonylbenzoyl adenosine; MANT-ATP, 3′(2′)-*O*-(methylantranoyl) adenosine 5-triphosphate; MANT-ADP, 3′(2′)-*O*-(methylantranoyl) adenosine 5′-diphosphate; PLP-ADP, pyridoxal 5′-diphosphoadenosine; PLP-AMP, pyridoxal 5′-monophosphoadenosine; TNP-ATP, 2′(3′)-*O*-(2,4,6-trinitrophenyl) adenosine 5′-triphosphate; TNP-ADP, 2′(3′)-*O*-(2,4,6-trinitrophenyl) adenosine 5′-diphosphate; thio-dATP, 2′-deoxyadenosine 5-*O*-(1-thiotriphosphate).

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with the Golgi apparatus and lysosomes (Wang and Guidotti, 1998; Biederbick et al., 2004). However, a soluble NTDase has recently been reported in rat blood serum (Oses et al., 2004). In animal tissues, apyrase is an ecto-enzyme with extracytoplasmic sites, thus regulating the extracellular levels of di- and triphosphorylated nucleosides (Bakker et al., 1994; Plesner, 1995; Schulte am Esch et al., 1999). Because of this ectolocalization, apyrase function has been involved in the regulation of cellular transduction pathways, the modulation of neural cell activity, the prevention of intravascular thrombosis, protein glycosylation, sugar level control, the regulation of membrane integrity, cholesterol transport and progesterone synthesis (Koziak et al., 2000; Flores-Herrera et al., 2002).

In plant tissues, apyrase can be a cytosolic, membrane-bound or nuclear protein (Valenzuela et al., 1989; Kettlun et al., 1992a; Komoszynski, 1996; Hsieh et al., 2000). It also seems to play many roles in plant such as: response to tactile stimuli, phosphate nutrition, and pollen germination. In legume roots, it is a nod factor that binds lectins that permit the establishment of rhizobium-legume symbiosis (Ghosh et al., 1998; Roberts et al., 1999; Thomas et al., 1999, 2000; Navarro-Gochicoa et al., 2003; Steinebrunner et al., 2003). In *Arabidopsis thaliana*, several important functions have been proposed for apyrase, such as connection with herbicide tolerance; a reduction of the ATP-dependent inhibitory effect on gravitropism associated with auxin transport; and thirdly, extracellular ATP regulation that activates signaling pathways triggered by Ca^{2+} (Windsor et al., 2003; Tang et al., 2003; Jeter et al., 2004).

In potato tuber, in particular, a function of apyrase in the biosynthesis of starch and cell wall has been suggested based on its capacity of hydrolyzing NDP (Valenzuela et al., 1989; Anich et al., 1990), while the enzyme localized in the nucleus would be important in controlling the intranuclear levels of dNTPs (Hsieh et al., 2000). This study is focused on isoapyrases purified from two potato tuber clonal varieties sharing the same molecular weight (49 kDa); similar proportion of acid and basic aminoacid residues; and possible essential amino acids (Kettlun et al., 1982; Mancilla et al., 1984). However, they differ in their isoelectric points and ATPase/ADPase ratios, Desirée enzyme has a p*I* of 6.69 and a hydrolysis ratio of approximately one; and Pimpernel apyrase, a p*I* 8.74 and a ratio of ten (Kettlun et al., 1982). In another potato variety, Ultimus, we have found the coexistence of at least two isoapyrases one with high and another with low ATPase/ADPase ratio. These isoenzymes also show some physical–chemical differences (Kettlun et al., 1992a,b). Although different apyrases in legumes, peas and soybean have been associated to different genes (Day et al., 2000; Shibata et al., 2001; Navarro-Gochicoa et al., 2003; Cannon et al., 2003), it is unknown whether the two potato isoapyrases in Ultimus correspond to different genes or to alternative splicing, as reported for animal tissues (Biederbick et al., 2000). Handa and Guidotti (1996) have isolated a single cDNA from a potato tuber library, but they indicated that Southern analysis suggested the existence of multiple genes.

The legume *Medicago truncala* contains from four to six-apyrase-like genes (Cohn et al., 2001; Navarro-Gochicoa et al., 2003), while two different genes have been identified in *Pisum sativum* and *Arabidopsis* (Shibata et al., 2001; Steinebrunner et al., 2003). In peas, one of the genes is more relevant in pollen tube germination (Apy2), whereas in seedling growth, the other gene (Apy1) is more important, being expressed as five different isotypes with characteristic p*I* values from p*I* 5.8 to 6.8 (Abe et al., 2002; Moustafa et al., 2003). These different isotypes have been attributed to post-traductional modification such as phosphorylation, a statement supported by the presence of phosphorylated apyrase in the brain (Wink et al., 2000), and also because the C-terminus of a recombinant pea nuclear apyrase is a substrate of casein kinase II (Hsieh et al., 2000). Apyrase with 49 kDa was present only after germination, and the abundance of the isotypes changed during the germination stage and in the various tissues. Since these isotypes vary in their enzymatic properties, it has been proposed that the differential expression of apyrase may play an important role in the early stage of germination and differentiation (Moustafa et al., 2003). During potato tuber growth, we found that apyrase activity was initially high and – after a significant diminution – reached a maximum on day 106, decreasing progressively until maturity (140 days) (Anich et al., 1990). No different apyrase isotypes were observed during potato growth.

Previous substrate specificity studies have shown that replacement of any of the oxygen atoms of ATP or ADP pyrophosphate bonds by a methylene-group did not prevent binding, resulting in competitive inhibitors (Del Campo et al., 1977; Kettlun et al., 1982). We were interested in studying the effect of modifications both in the ribose (deoxyribonucleotides, TNP- and MANT-derivatives) and the base (ethene-derivatives) moieties on the catalytic efficiency in order to determine the influence of the different portions of the substrate on binding to the active site of apyrase (see Fig. 1, compounds **1**–**3**).

Affinity labeling has been extensively utilized to elucidate the sequence of substrate binding site using analogues that form stable covalent bonds with some amino acid residues. Several nucleotide analogues have been successfully used as affinity labels for animal apyrases and some ATPases including azido-nucleotides reacting with lysine, carboxylic and thiol groups (Lebel and Beattie, 1986; Martí et al., 1997); and PLP-derivatives of nucleotide and FSBA labeling lysine residues (Tamura et al., 1986; Fukui, 1995; Sévigny et al., 1995, 1997; Martí et al., 1996, 1997; Torres et al., 1998).

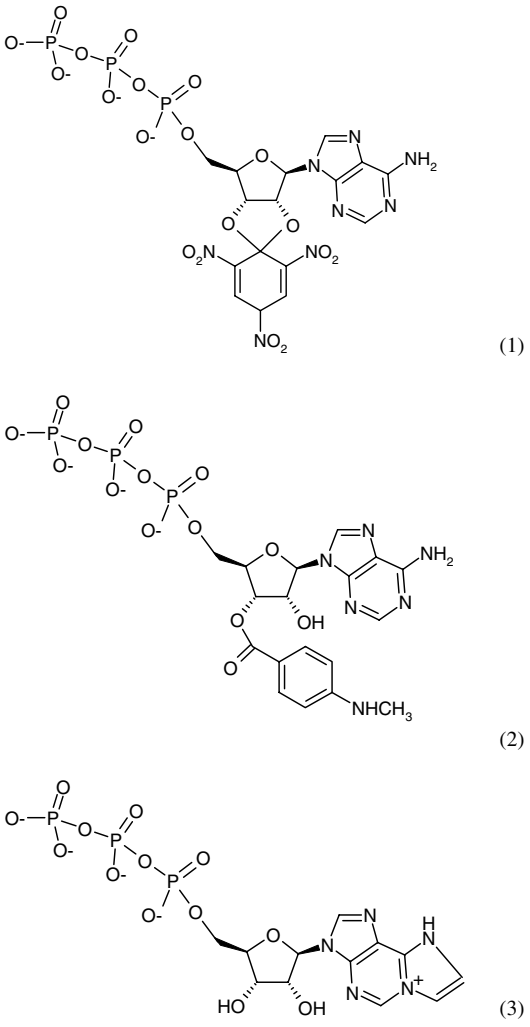

Fig. 1. Chemical structures of fluorescent ATP derivatives: TNP-ATP (**1**); MANT-ATP (**2**); Etheno-ATP (**3**).

We compared both isoenzymes in relation to their efficiency on different deoxynucleotides and nucleotide analogues modified either in the ribose, the base moiety, or the phosphate group (O atom substituted by S) (See Fig. 1, Compounds **1**–**3**). For future sequencing studies, we also explored (a) the possibility of modifying the nucleotide binding site with affinity labeling compounds such as PLP- and azido-nucleotides, and FSBA, and (b) characteristic proteolytic patterns in SDS/PAGE after digestion of both isoenzymes with proteases such as trypsin, chymotrypsin and Glu-C.

2. Results and discussion

2.1. Determination of possible contaminant activities of the commercially available enzymes

The study of the kinetic parameters of enzymes requires preparations free from contaminant activities that use common substrates, thus producing altered kinetic parameters. On the other hand, affinity labeling experiments required pure enzymes. Therefore, we measured the presence of some artefactual activities such as 5′ nucleotidase, alkaline and acid phosphatase activities in the apyrases purified in our laboratory (showing a single protein band by SDS/PAGE, Espinosa et al., 2000, 2003); and in two commercial preparations with high and low ATPase/ADPase ratios, respectively. Commercial apyrase with high ATPase/ADPase ratio of 6.6 showed 1240 and 190 U/mg protein of ATPase and ADPase, respectively, and the low ratio enzyme of 0.96 contained 11.4 and 11.8 U/mg of ATPase and ADPase, respectively. Phosphatase and nucleotidase activities were absent in our preparations, but in commercial apyrases, we detected 19.6 and 0.12 U/mg of alkaline phosphatase in the high and low ratio enzymes, respectively, and the low ratio apyrase additionally showed 3.1 U/mg of acid phosphatase activity. These results indicate the uselessness of doing labeling and sequencing studies with commercial enzymes.

2.2. Determination of the kinetic parameters K_m and k_{cat}

Table 1 shows that all nucleotide analogues tested were cleaved by both Desirée and Pimpernel enzymes. Di- and triphosphorylated nucleotide analogues were modified in different parts of the molecule: deoxyribonucleotides (dATP, dGTP, dTTP and dCTP), MANT- and TNP- derivatives were modified in the ribose, while the ethene-derivatives, in the base. We also tested thio-substituted dATP (one α oxygen–phosphorus substituted by sulphur), widely used as dATP analogue. Pimpernel enzyme keeps high NTPase/NDPase ratio as its main feature while Desirée maintains a low activity ratio with all the tested nucleotides.

Modifications in the base, such as the inclusion of an ethene group, did not alter the kinetic parameters of apyrases (Table 1). However, modifications in the ribose produced differential effects on both isoapyrases. Except for MANT-ATP (**2**) in Pimpernel apyrase, the substitution with the two bulky groups, TNP- and MANT, produced reductions in K_m and k_{cat} values. These changes in kinetic parameters account for the differences in the catalytic efficiency (k_{cat}/K_m) of both isoenzymes, the efficiency of the NDPase activity of Pimpernel enzyme being more affected, increasing from 4.7 to 26-fold with MANT- and TNP- modification, respectively.

Deoxyribose replacement of ribose produced better substrates for Desirée enzyme with higher k_{cat} and catalytic efficiency values, but with similar affinity (K_m) like ATP-containing ribose sugar. In the case of Pimpernel, only slight differences in k_{cat} were found; however, changes in K_m led to large differences in catalytic efficiency. Assuming that K_m is a parameter that correlates with the affinity of apyrases for substrates, the current

Table 1
Kinetic parameters for Desirée and Pimpernel apyrases

Nucleotide	Desirée enzyme			Pimpernel enzyme		
	K_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($M^{-1}\ s^{-1}$)	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($M^{-1}\ s^{-1}$)
ATP	1685[a]	0.025[a]	67.4×10^6	700[a]	0.060[a]	11.7×10^6
ATP	1439[a]	0.070[a]	20.6×10^6	79[a]	0.250[a]	0.3×10^6
ε-ATP(**3**)	1642	0.031	53.0×10^6	682	0.024	28.4×10^6
ε-ADP	1384	0.073	20.0×10^6	86	0.114	0.8×10^6
TNP-ATP(**1**)	982	0.018	55.0×10^6	191	0.008	23.9×10^6
TNP-ADP	328	0.019	17.3×10^6	70	0.009	7.8×10^6
MANT-ATP(**2**)	965	0.012	80.4×10^6	800	0.018	44.4×10^6
MANT-ADP	828	0.014	59.1×10^6	23	0.017	1.4×10^6
dATP	2222	0.018	123.4×10^6	636	0.031	20.5×10^6
dGTP	3019	0.028	107.8×10^6	573	0.133	4.3×10^6
dTTP	2841	0.027	105.2×10^6	455	0.093	4.9×10^6
dCTP	2174	0.029	75.0×10^6	490	0.032	15.3×10^6
Thio-dATP	1101	0.021	52.4×10^6	595	0.048	12.4×10^6

[a] From Kettlun et al. (1982).

results suggest a high affinity towards any nucleotidic base, inferred from comparing with our previous results on several synthetic substrates (Del Campo et al., 1977). In them, we found that the nucleotidic base replacement by several organic groups produced a significant increase in K_m. This is the first study on the effects of ribose modifications, indicating that the introduction of bulky groups (MANT- and TNP-) does not produce steric hindrance on substrate binding. However, except for MANT-ATP (**2**) in the case of Desirée, a significant reduction in k_{cat} was found, which indicates an important effect on the catalytic step. Finally, the replacement of oxygen with sulfur atom did not affect binding, but reduced the catalytic action. Differences in the specificity and catalytic effect on NDP and NTP of these two enzymes could be explained by differences in amino acid residues of the active site. This is supported by experiments done on animal apyrases including mutations of certain amino acid residues, chimeric constructions or splice variants where the characteristic ATPase/ADPase ratio could be altered depending on the case (Hicks-Berger and Kirley, 2000; Biederbick et al., 2000; Heine et al., 2001). Substitution of two specific histidine residues (Grinthal and Guidotti, 2000, 2002), and mutagenesis of a specific asparagine, glutamine, and arginine residues, rendered an apyrase with higher ADPase than ATPase activity (Yang et al., 2001). On the other hand, the change of another arginine residue by proline or tryptophan by alanine gave origin to NTPase activity (Smith et al., 1999; Yang et al., 2001).

In order to study the binding site, we tried to label potato apyrase with some compounds reported as affinity labels for several nucleotide binding-enzymes including apyrase of animal origin. We expected to obtain labeled peptides corresponding to the active site of both isoapyrases for further sequencing studies.

2.3. Treatment of isoapyrases with nucleotide affinity labels

Photoreactive analogues of ATP and ADP, 8-azido-derivatives used as affinity labeling of adenine binding sites (Lebel and Beattie, 1986; Martí et al., 1997) were hydrolyzed by both apyrases when tested in the dark. However, when activated with UV light these compounds only produced about 10% inactivation of Desirée apyrase with some higher effect (15–23%) in Pimpernel. Several publications report on the formation of covalent bonds in apyrases from animal sources with a region near to the substrate-azide group through lysine, threonine or cysteine residues (McIntosh et al., 1992; Salvucci et al., 1994). These studies include apyrases from pancreatic zymogen granule membranes, chromaffin cells, and presynaptic plasma membrane preparations from Torpedo electric organ that underwent photoaffinity labeling using 8-azido-ATP and 8-azido-ADP at micromolar concentrations (up to 100 μM) with an activity loss between 40% and 60% (Lebel and Beattie, 1986; Rodríguez-Pascual et al., 1993; Martí et al., 1997). These nucleotide derivatives should bind to the active site because in the absence of light they were hydrolyzed by these apyrases, and also are competitive inhibitors of ATP and ADP. However, the low inhibitory effect using a high concentration (256 μM) discards its use as an adequate affinity label for Desirée and Pimpernel apyrases.

Another nucleotide analogue widely utilized as an affinity label for apyrases from different animal sources is FSBA (Sévigny et al., 1995, 1997; Martí et al., 1996; Torres et al., 1998; Flores-Herrera et al., 2002). Although we expected similar results with potato tuber apyrases, only Desirée apyrase showed some extent of inactivation (15–30%) with 10 mM FSBA, a concentration several

magnitude orders higher than the micromolar concentrations required for animal apyrase inactivation. Preincubation of Desirée apyrase with 10 mM ATP or ADP phosphonates prior to addition of the modifier, did not prevent from inactivation. FSBA has been described as an efficient affinity label for proteins with an ATP binding site, including substrate protection from inactivation (Sévigny et al., 1995; Torres et al., 1998). Changes in incubation conditions such as pH (6.0, 7.4, and 8.0), and presence or absence of bivalent cations (Mg^{2+} or Ca^{2+}), did not improve the inhibitory effect of FSBA. Considering the low inactivating effect and the lack of substrate protection, we can propose that potato apyrase active site is quiet different from animal apyrases.

Finally, other ATP and ADP nucleotide analogues reported as modifiers of lysine through a Schiff base, are pyridoxal nucleotides (Tamura et al., 1986; Fukui, 1995; González-Nilo et al., 2000). Binding these analogues to the nucleotide active site can produce inactivation, which is useful to obtain information about the structure of different enzymes with adenine and NAD binding sites. However, both apyrases did not react with these nucleotide analogues at 53 μM concentration. According to our previous data showing that any modification in the diphosphoric chain prevents catalysis, it is possible that the presence of PLP-moiety does not allow binding of these compounds to the proteins because of steric hindrance (Del Campo et al., 1977).

The modest inactivation or the lack of effect produced by FSBA, 8-azido-nucleotides and PLP-derivatives, discard the proximity or adequate orientation of sulfhydryl, hydroxyl, or amino groups in the active site. The lack of modifications of sulfhydryl and hydroxyl groups in potato isoapyrases has discarded their essentiality (Kettlun et al., 1982, 1992b; Valenzuela et al., 1989). The catalytic importance of amino groups has not been considered since the inactivation rate with 1,3,5-trinitrobenzenesulfonic acid is slower than the modification rate at A_{340nm}. Therefore, these compounds are not suitable for binding site labeling.

2.4. Proteolytic susceptibility of both isoapyrases under native and denaturing conditions

This study was initiated as part of the classical isolation of active site affinity labeled peptides. Partial hydrolysis by trypsin, chymotrypsin and endoprotease Glu-C were performed. Under native conditions, apyrase was not hydrolyzed by trypsin, but chymotrypsin and Glu-C showed a differential proteolytic effect depending on the isoenzyme. After 1 h of incubation, Desirée native apyrase showed two new protein bands of M_r 24 and 20 kDa, while Glu-C treatment took at least 20 h to produce two protein bands of 24 and 18 kDa. Almost no change in the 49 kDa band was observed in both treatments (data not shown). On the other hand, Pimpernel enzyme was more labile to proteolysis with these proteases with a continuous disappearance of the 49 kDa band during the 20-h incubation without visualization of lower M_r bands (data not shown).

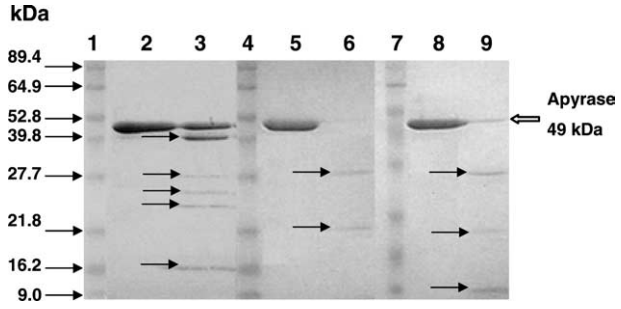

Fig. 2. Proteolytic pattern of Desirée apyrase. Portions of Desirée enzyme before (lanes 2, 5 and 8) and after 1 h of proteolytic treatment at 37 °C with Glu-C (lane 3), chymotrypsin (lane 6), and trypsin (lane 9), together with M_r standards (lanes 1, 4 and 7), submitted to SDS–PAGE at 12% polyacrylamide.

Partial denaturation of Desirée and Pimpernel apyrases with 4 M urea made them susceptible to the three proteases, the 49 kDa band decreasing with the concomitant appearance of peptides with lower M_r. In Fig. 2, as an example of hydrolysis, we show the Glu-C treatment of Desirée enzyme where at least five peptides with M_r of 40, 28, 25, 23, and 16 kDa were obtained. Chymotrypsin treatment produced 32 and 22 kDa peptides, while trypsin incubation showed the formation of 24, 19 and 9 kDa peptides. Therefore, Glu-C proteolysis would be more appropriate for labeled peptides isolation from Desirée apyrase. However, in the case of Pimpernel enzyme, we only observed the production of two peptides with M_r of 36 and 33 kDa with Glu-C, 33 and 18 kDa with chymotrypsin, and 35 and 22 kDa with trypsin (not shown).

These results imply a different degree of exposure of Arg and Lys (trypsin treatment), Phe, Try and Trp (chymotrypsin treatment), Glu and Asp (Glu-C treatment) in both enzymes either in their native or partially denatured forms (4 M urea).

3. Concluding remarks

Structural differences between Pimpernel and Desirée apyrases account for (1) different observed proteolytic patterns since, in spite of their similarity in amino acid composition, their sequence should yield a distinct three-dimensional structure; (2) differences in the hydrolytic activity ratio on tri- and diphosphorylated derivatives. Modifications on the base or the ribose did not produce significant effects on K_m of both isoenzymes, suggesting that introduction of bulky groups (MANT- and TNP-) does not produce steric hindrance on

substrate binding. The lack of sensitivity of potato apyrases towards FSBA or azido-nucleotide labeling agrees with the substantial differences between the potato and animal enzymes.

4. Experimental

4.1. Materials

TNP-ATP (**1**), TNP-ADP, ε-ATP, ε-ADP and methylisatoic anhydride were obtained from Molecular Probes Inc. (Eugene, OR, USA); ATP, ADP and FSBA were purchased from Sigma Chemical Co. (St Louis, MO, USA); dNTP set of 2′deoxynucleoside 5′-triphosphate were from Amersham-Pharmacia Inc. (Piscataway, NJ, USA) and thio-ATP was from PerkinElmer, Life and Analytical Sciences (Beaconsfield, UK).

4.2. Synthesis of nucleotide analogues

MANT-ATP (**2**) and MANT-ADP were synthesized and purified as previously described (Hiratsuka, 1983). 8-azido-ATP derivatives were prepared as described in Schäfer et al. (1978), and PLP-AMP and PLP-ADP according to Tamura et al. (1986).

4.3. Methods

4.3.1. Apyrase purification

Apyrase was purified from homogeneous strains of *S. tuberosum* cv Pimpernel and Desirée, as previously reported (Espinosa et al., 2000, 2003).

4.3.2. Enzymatic activities

Apyrase activity was assayed at 30 °C by measuring the release of inorganic phosphate (Pi) from ATP or ADP using the method described elsewhere (Chen et al., 1956). The assay medium contained 0.1 M MES pH 6.0 and 5 mM $CaCl_2$ (Espinosa et al., 2000). For kinetic parameters: determinations were performed measuring released Pi according to Ernster et al. (1950). The concentration range of the nucleotide analogues was between 0.01 and 0.18 mM. Kinetic parameters were calculated from Hanes-Woolf plots by the least square method showing a linear correlation ranging from 0.987 to 0.99. The 5′ nucleotidase activity was assayed using 2 mM AMP in the presence of 5 mM $MgCl_2$ in 40 mM Tris–HCl pH 7.5, and Pi was determined according to Ernster et al. (1950). Alkaline and acid phosphatases were followed by direct spectrophotometric measurements at 405 nm with 5 mM *p*-nitrophenylphosphate in 40 mM Tris–HCl pH 9.0 for the alkaline enzyme, and in 40 mM sodium citrate pH 4.8 for acid phosphatase.

For all kinetic analyses, the fraction of nucleotide used ranged from 5% to 20%, showing a linear response for a 10 min assay. A concentration of 0.06 nM of apyrase was used for phosphoderivatives assays, except for diphosphoderivative assays with Pimpernel enzyme where a 0.6 nM concentration was required.

4.3.3. Protein determinations

Protein was determined by the Lowry method, using bovine serum albumin as standard (Lowry et al., 1951).

4.3.4. Affinity labeling of isoapyrases

(a) *Photoinactivation with 8-azido-ATP*: 20 μg of apyrase was treated with 250 μM 8-azido-ATP in 0.1 M MES pH 6.0, and irradiated for 8 min at 0 °C with a longwave UV lamp Black-Ray B-100A, using a 10-cm-distance. Irradiated enzymes in the absence of 8-azido-ATP were used as controls. (b) *Reaction with PLP-AMP and PLP-ADP derivatives*: 6–12 μg of apyrase were incubated with 50 μM of PLP-derivative dissolved in EtoH in the presence of 50 μM Tris–HCl pH 8.0 and 1 mM EDTA. The control included EtoH(10%) instead of PLP-derivatives. Incubations were done at 30 °C for 60 min. The reaction was stopped by dilution of the reaction medium 50- or 100-fold with 10 mM sodium borohydride. (c) *Inactivation with FSBA*: 250 μg of Desirée or Pimpernel apyrase were preincubated from 0 to 15 min at 37 °C in a medium with 150 mM KCl, 5 mM $MgCl^2$, 50 mM sodium acetate, 50 mM de Tris pH 7.4 and 10 mM FSBA dissolved in DMSO; this solvent was used as a control. This assay was also performed at pH 6.0, 7.4 and 8.0 in the presence or absence of bivalent cations (5 mM Mg^{2+} or Ca^{2+}). The reaction was stopped by dilution of the reaction medium 30- or 50-fold in 0.1 M NaCl before measuring apyrase activity. Substrate protection was only tested for FSBA inactivation of Desiree apyrase, preincubating the enzyme with 10 mM ATP or ADP phosphonate previous addition of the modifier.

4.3.5. Proteolytic digestion of apyrases

Homogeneous fractions of 0.05 mg of Pimpernel and Desiree enzymes were treated during 1, 4, 8 and 20 h at 37 °C with 0.01 mg/ml of the following proteolytic enzymes: (a) chymotrypsin, (b) endoprotease Glu-C, and (c) trypsin. The incubation medium in all the cases was 50 mM Tris–HCl pH 8.0. Experiments were performed both in the presence and absence of 4 M urea. Proteolytic patterns were followed by SDS–PAGE at 12% (Laemmli, 1970).

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