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Differences in nucleotide-binding site of isoapyrases deduced from tryptophan fluorescence

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Abstract

Comparative studies of intrinsic and extrinsic fluorescence of apyrases purified from two potato tuber varieties (Pimpernel and Desirée) were performed to determine differences in the microenvironment of the nucleotide binding site. The dissociation constants (K_d) of Pimpernel apyrase for the binding of different fluorescent substrate analogs: methylanthranoyl (MANT-), trinitrophenyl (TNP-), and ε-derivatives of ATP and ADP were determined from the quenching of Trp fluorescence, and compared with K_d values previously reported for Desirée enzyme. Binding of non-fluorescent substrate analogues decreased the Trp emission of both isoapyrases, indicating conformational changes in the vicinity of these residues. Similar effect was observed with fluorescent derivatives where, in the quenching effect, the transfer of energy from tryptophan residues to the fluorophore moiety could be additionally involved. The existence of energy transfer between Trp residues in the Pimpernel enzyme was demonstrated with ε-analogues, similar to our previous observations with the Desirée. From these results we deduced that tryptophan residues are close to or in the nucleotide binding site in both enzymes. Experiments with quenchers like acrylamide, Cs^+ and I^-, both in the presence and absence of nucleotide analogues, suggest the existence of differences in the nucleotide binding site of the two enzymes. From the results obtained in this work, we can conclude that the differences found in the microenvironment of the nucleotide binding site can explain, at least in part, the kinetic behaviour of both isoenzymes.

Keywords: *Solanum tuberosum*; Solanaceae; ATP-diphosphohydrolase; Potato apyrase; Desirée and Pimpernel isoapyrases; Intrinsic and extrinsic fluorescence

1. Introduction

Apyrase (ATP-diphophohydrolase, E.C. 3.6.1.5) catalyses the cleavage of ATP and ADP with liberation of orthophosphate (Traverso-Cori et al., 1965). In animal tissues it is located as an integral membrane protein and its function is related to the control of the extracellular concentration of di- and triphosphorylated nucleosides (Plesner, 1995; Komoszynski, 1996; Zimmermann et al., 1998; Guranowski, 2000; Gendron et al., 2000; Heine et al., 2001; Boeck et al., 2002), and to its decrease during apoptosis in response to high concentrations of extracellular ATP (Goepfert et al., 2000). The transmembrane domain of ectoapyrase affects its activity and quaternary structure (Wang et al., 1998; Grinthal and Guidotti, 2002). In plant tissues it has been described in the cytosolic fraction (Valenzuela et al., 1989; Kettlun et al., 1992a), nucleus (Hsieh et al., 2000) and membrane fractions (Tognoli and Marré, 1981; Vara and Serrano, 1981; Valenzuela et al., 1989; Kettlun et al., 1992a; Day et al., 2000). With this ubiquitous localization, plant apyrases have been involved in responses to tactile stimuli (Ghosh et al., 1998a,b), recognition of exogenous carbohydrates (Etzler et al., 1999), phosphate nutrition (Thomas et al.,

Abbreviations: MANT-ATP, 3′(2′)-*O*-(methylantranoyl) adenosine 5′-triphosphate; MANT-ADP, 3′(2′)-*O*-(methylantranoyl) adenosine 5′-diphosphate; TNP-ATP, 2′(3′)-*O*-(2,4,6-trinitrophenyl) adenosine 5′-triphosphate; TNP-ADP, 2′(3′)-*O*-(2,4,6-trinitrophenyl) adenosine 5′-diphosphate; ε-ATP, 1,N^6-ethenoadenosine triphosphate; ε-ADP, 1,N^6-ethenoadenosine diphosphate; ADP-PCP, adenosine 5′-(β,γ-methylene) triphosphate; AMP-PCP, adenosine 5′-(α,β-methylene) diphosphate

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1999), legume nodulation (Day et al., 2000) and xenobiotic resistance (Roberts et al., 1999; Thomas et al., 2000).

In potato tuber, this enzyme exists in more than one molecular form depending upon the clonal variety (Traverso-Cori, et al., 1970; Kettlun et al., 1982; Mancilla et al., 1984; Kettlun et al., 1992a,b). Apyrases from different clonal sources exhibit differences both in isoelectric point and relative rate of hydrolysis of ATP and ADP (Traverso-Cori et al., 1970; Kettlun et al., 1982, 1992a,b). With the purpose of understanding the observed kinetic differences, we have chosen as models an apyrase purified from *Solanum tuberosum* var. Pimpernel (Pimpernel apyrase) with ATPase/ADPase ratio close to 10 (Del Campo et al., 1977; Kettlun et al., 1982), and an apyrase isolated from the Desirée variety (Desirée apyrase) with an ATPase/ADPase ratio close to 1.0 (Kettlun et al., 1982). By chemical modification, both apyrases appear to have the same enzymatically significant amino acid residues; thus, the question of the observed kinetic differences remains unsolved (Kettlun et al., 1982, 1992b). Some site-directed mutagenesis experiments have been performed with the human enzyme. The results indicate that some conserved glycine, histidine, asparagine and glutamine, and a cysteine in the C-terminal region are important for determining substrate specificity, and the replacement of some of them produce changes in the ATPase/ADPase ratio (Grinthal and Guidotti, 2000; Wink et al., 2000a; Heine et al., 2001; Yang et al., 2001; Hicks-Berger et al., 2001; Kirley et al., 2001). Brain ecto-apyrase has been detected as a phosphoprotein (Wink et al., 2000b).

We have reported extrinsic fluorescence studies with Desireé apyrase, using several fluorescent nucleotide analogues with the purpose of examining the molecular topography in the nucleotide-binding region of the protein (Espinosa et al., 2000). In those experiments, strong quenching of Trp fluorescence upon binding to nucleotide analogues, either with or without fluorescent moieties, was observed. An increase in the fluorescence was found with all the fluorescent analogues tested. This enhancement in the Desirée enzyme could be attributed to the binding of the fluorescent portion of the nucleotide to hydrophobic regions, and/or to energy transfer from the indole chromophore donor (Trp) to the nucleotide analogue acceptor, suggesting that at least some of the Trp residues are located in close proximity to the nucleotide binding site.

Fluorescence of the indole chromophore is highly sensitive to the environment, being a useful tool to detect protein conformational changes and to follow the interaction of a protein with ligands and substrates (Chen and Barkley, 1998). In the present work, we report comparative fluorescence studies of the high (Pimpernel apyrase) and low (Desireé apyrase) ATPase/ADPase ratio apyrases which may help understand the different properties of the two apyrases.

2. Results and discussion

2.1. Quantification of the number of Trp residues of Pimpernel apyrase

Amino acid analysis ($n=3$) after acid hydrolysis of Pimpernel apyrase showed the presence of 3.8 ± 0.6 Trp residues. This value is comparable to 4.17 ± 0.19 Trp residues previously reported for Desirée apyrase (Espinosa et al., 2000), and suggests that both enzymes contain the same number of Trp residues.

2.2. Intrinsic fluorescence of Pimpernel and Desirée apyrases and quenching effects

Previous studies of apyrase intrinsic fluorescence emission have shown that the intrinsic fluorescence of Trp in each apyrase was different in two aspects: (a) maximum emission wavelengths of the Desirée and Pimpernel enzymes were 336 and 340 nm, respectively, suggesting small differences in the microenvironment of Trp residues; and (b) although both enzymes have the same number of tryptophans, the intensity of fluorescence emission of the Pimpernel enzyme doubled that of Desireé apyrase (Kettlun et al., 2000). Binding of a non-hydrolysable nucleotide analogue (AMP-PCP) decreased the Trp fluorescence intensity of both isoapyrases, without changes in the emission maximum wavelength (Kettlun et al., 2000). Similar results were observed upon ADP-PCP binding. This decrease in fluorescence emission suggests conformational changes in the vicinity of Trp residues upon nucleotide binding (Kettlun et al., 2000). The similar emission maximum for free and ligand-bound apyrase suggests that these conformational changes do not expose Trp residues to more hydrophilic environments.

The effect of quenchers on the intrinsic fluorescence of proteins can be used to infer the relative disposition of Trp residues in the tertiary structure, as well as the effect of ligand binding on that distribution (Lakowitz, 1983). To discriminate between different populations of Trp residues in Pimpernel and Desirée apyrases, three quenchers of different polarity were used: acrylamide, Cs^+, and I^- (Ferreira, 1993) Acrylamide, a polar uncharged quencher, can probably quench both exposed and unexposed residues, although it has a smaller degree of penetration than O_2 (Eftinck and Ghiron, 1981). Cs^+ and I^- are two oppositely charged quenchers that may collide with exposed indole groups, and also with those groups located in a negative or positive environment, respectively.

Stern-Volmer plots for both Pimpernel and Desirée apyrases are somewhat different. Acrylamide quenching of Pimpernel showed a linear Stern-Volmer plot indicating that all Trp residues are equally quenched by acrylamide (Fig. 1A), while Desirée apyrase showed an

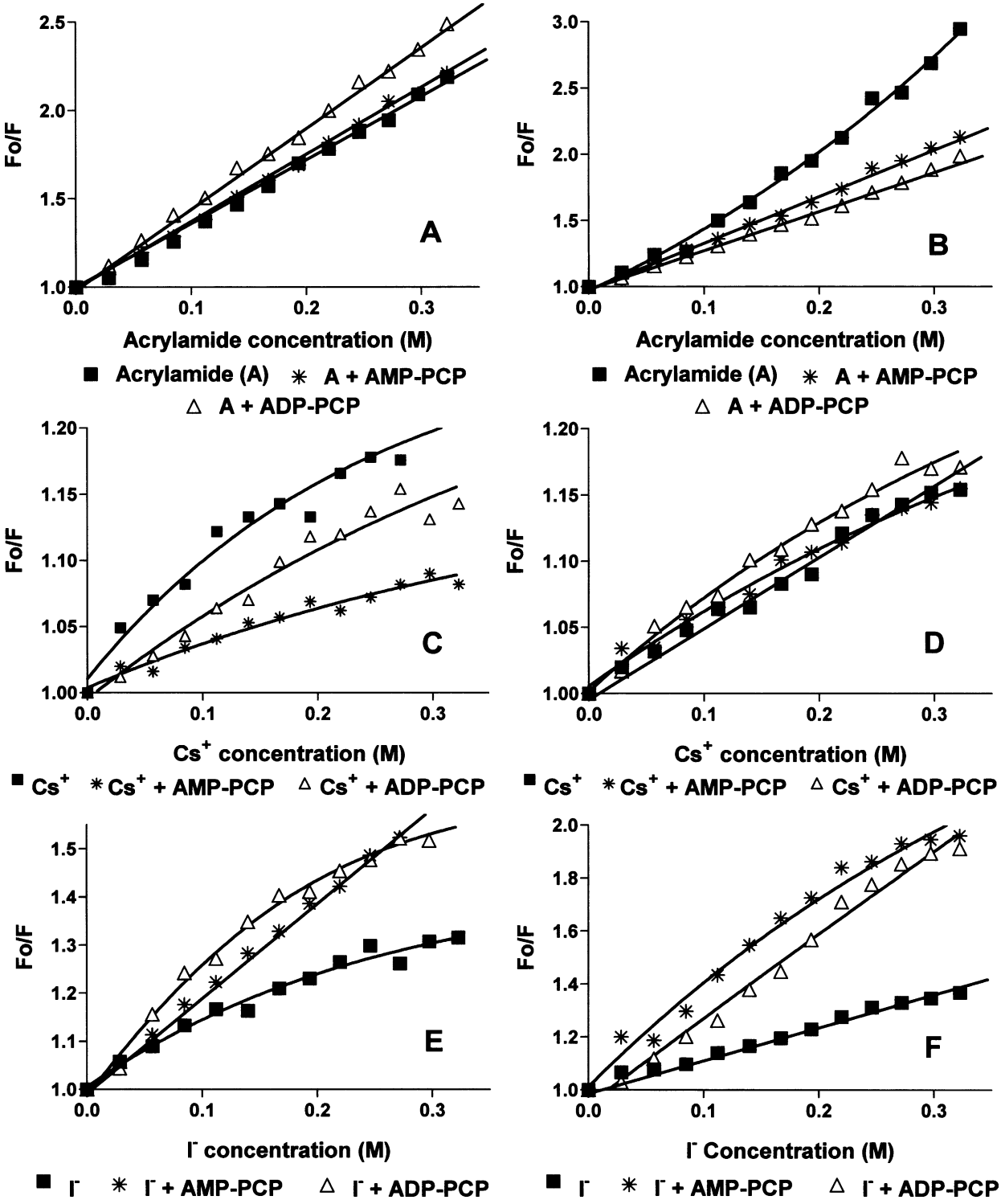

Fig. 1. Stern-Volmer plots of quenching of the apyrases in the presence and absence of AMP-PCP and ADP-PCP. A, C and E: Pimpernel enzyme; B, D and F: Desirée enzyme. Fo/F: emission of fluorescence ratio in presence or absence of quencher. The emission intensity was measured at 340 nm for Pimpernel and 336 nm for Desirée. Protein concentration was 0.06 mg/ml in 100 mM MES pH 6.0 for both enzymes. Nucleotide analogues were 2 mM. Corrections were made for dilution upon quencher addition. Data are means of at least three independent experiments.

upward curvature, pointing to some contribution of static quenching (Fig. 1B) (Lakowitz, 1983). When Cs^+ or I^- were employed, downward curvatures were obtained for Pimpernel apyrase (Fig. 1C and E), indicating that some Trp do not collide with these quenchers (Eftinck and Ghiron, 1981). This lower Trp accessibility to charged quenchers evidenced in the Pimpernel apyrase suggests that some Trp residues are in hydrophobic regions. For the Desirée enzyme, Stern-Volmer plots with the two charged quenchers resulted in straight lines

(Fig. 1D and F), suggesting that all Trp residues are accessible to ionic quenchers of different charge.

The effect of non-hydrolyzable substrate analogues, ADP-PCP and AMP-PCP on the quenching of Trp fluorescence of the two apyrases was also investigated. In the Pimpernel enzyme an increase in the Trp accessibility to acrylamide was seen upon ADP-PCP binding, with no changes in the presence of AMP-PCP (Fig. 1A). The upward curvature of the plot observed with Desirée apyrase was supressed with a decrease in quencher accesibility in the presence of substrate analogues (Fig. 1B). These results suggest conformational changes induced by ligand binding that modify dipolar contributions around Trp residues located near the active site.

When these experiments were carried out using Cs^+, Pimpernel apyrase maintained the downward curvature of the Stern-Volmer plot in the presence of either of the two analogues, with a decrease in the quencher accessibility (Fig. 1C). For Desirée apyrase (Fig. 1D), the linear Stern-Volmer plot obtained for the protein alone changed to a slightly downward curvature. This indicates that upon substrate analogue binding to this isoapyrase, the Trp residues which were equally accessible to the quencher in the absence of ligand, result in different populations, as a consequence of conformational changes.

Finally, when I^- was used as a quencher in the presence of ADP-PCP or AMP-PCP, the increased quenching effect on Trp fluorescence observed points to conformational changes upon ligand binding in both isoenzymes (Fig. 1E and F). Additionally, AMP-PCP binding produced different changes in plot curvatures; in the case of Pimpernel apyrase the curved line was transformed into a straight line, whereas in the Desirée, the straight line changed to a downward curvature. Considering that there is no shift in the maximum emission wavelength of both enzymes in the presence of nucleotide analogues, the larger accessibility to I^- quenching does not necessarily imply an increase in the solvent accessibility of indole groups.

2.3. Resonance energy transfer between Trp donors and ethene-derivatives in the Pimpernel apyrase

Chemical modification studies of Pimpernel and Desirée apyrases suggest the presence on Trp residues close to the nucleotide-binding site (Kettlun et al., 1982). Our previous report on energy transfer from Trp to ε-derivatives of ADP and ATP in Desirée isoapyrase supports this proposal (Espinosa et al., 2000). This observation implies that the donor and the acceptor are located at short distances in a suitable orientation for the energy transfer process to take place (O'Donoghue et al., 1992). The binding of ε-derivatives of ADP or ATP to Pimpernel apyrase produces: (a) an increase in the fluorescence emission of the probes, with no changes in the maximum emission wavelength, and (b) a decrease in Trp fluorescence. The quenching effect of ε-ATP on Pimpernel Trp fluorescence is shown in Fig. 2 (Part A). In Fig. 2 (Part B) the differential spectra of this ATP derivative bound to the enzyme (spectrum of ε-ATP in the presence of Pimpernel apyrase minus the spectrum of free ε-ATP) is presented. The increase in fluorescence emission suggests that the fluorescent portion of the analogue is relatively buried or rigidly positioned in the nucleotide-enzyme complex, because decreasing solvent polarity increases the quantum yield of the fluorophore (Lakowitz, 1983). The quenching of the intrinsic protein fluorescence points to either local changes in the environment of Trp and/or an efficient energy transfer from some of the Trp residues to the ϵ-nucleotide analogue (Bujalowski and Klonowska, 1994a,b; Espinosa et al., 2000). Similar results, involving quenching of intrinsic fluorescence and increasing of extrinsic fluorescence, were obtained upon binding of methylanthranoyl- (MANT-) and trinitrophenyl-(TNP-)derivatives of ADP and ATP (data not shown).

In analogy to our previous results with the Desirée enzyme (Espinosa et al., 2000), we have used the approach described by Bujalowski and Klonowska (1994b), where the excitation spectra of an acceptor (fluorescent nucleotide), in the presence of the donor (apyrase Trp), provides direct evidence of fluorescence energy transfer. Fig. 3 shows the excitation spectra ($\lambda_{em} = 413$ mn) of ε-ATP in the absence (curve b) or presence of Pimpernel apyrase (curve a). The spectra have been normalised at $\lambda_{ex} = 325$ nm, where there is no excitation of the protein indole groups, thus the change in the nucleotide fluorescence is due only to changes in their quantum yield produced upon enzyme binding (Bujalowsky and Klonoswska, 1994b). In the presence of apyrase, there is an additional increase in the nucleotide fluorescence intensity, centred at 286 nm. The difference between the two excitation spectra of the ε-ATP (with and without apyrase) coincides well with the excitation spectra of Pimpernel apyrase recorded at 340 nm (data not shown). In the spectral range 250–300 nm, the ε-derivatives do not absorb in the concentration range used, and apyrase alone does not show any emission at $\lambda_{ex} = 413$ nm. These results allow us to conclude that the increase in the emission of ε-nucleotides upon protein binding is due to energy transfer from Trp donors to the ϵ-nucleotide acceptor. It is possible to infer then, that the organic moiety of ε-ADP and ε-ATP binds in the neighbourhood of some of the four Trp residues of the enzyme. A similar conclusion was previously obtained for Desirée apyrase using an analogous approach (Espinosa et al., 2000).

Binding of the other fluorescent derivatives (TNP- and MANT-), which also present some overlapping

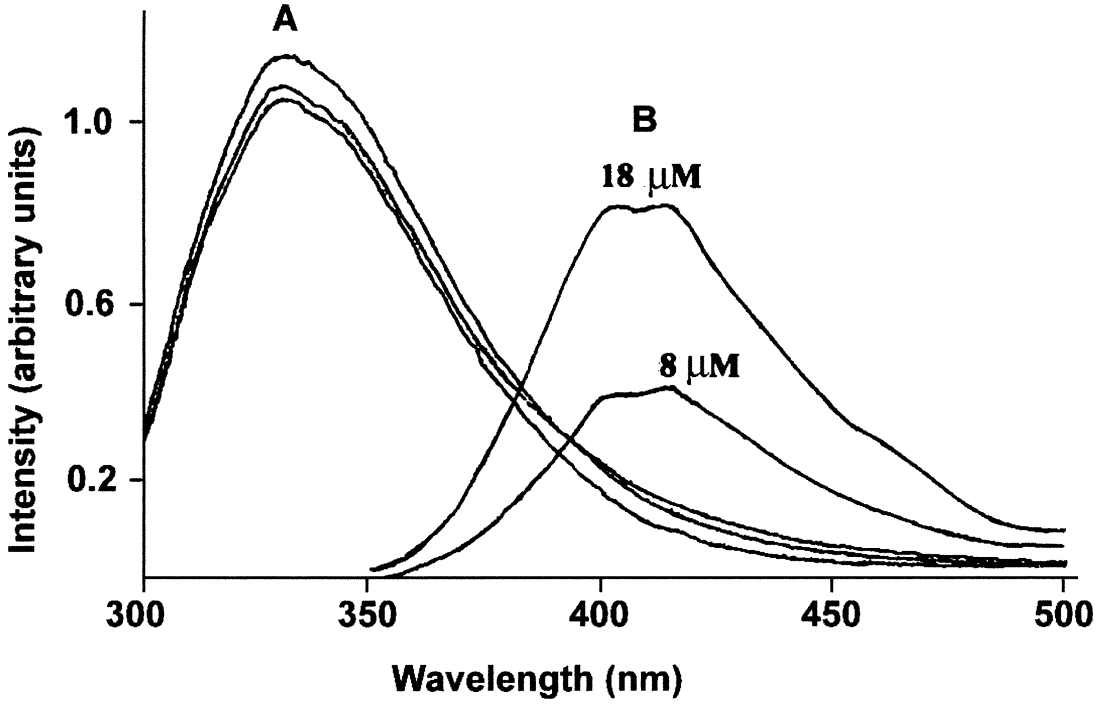

Fig. 2. Intrinsic and extrinsic fluorescence spectra of Pimpernel apyrase in the presence and absence of ε-ATP. (A) The left side plot represents the quenching of intrinsic fluorescence emission of apyrase by titration with ε-ATP (λ_{ex}: 286); (B) The right side plot corresponds to the differential emission spectra of ε-ATP (λ_{ex}: 325 nm) (spectrum of ε-ATP in the presence of Pimpernel apyrase minus the spectrum of free ε-ATP). Spectra were recorded in 100 mM MES buffer, pH 6.0, with two concentrations of nucleotide (8 and 18 μM), and 1.2 μM apyrase.

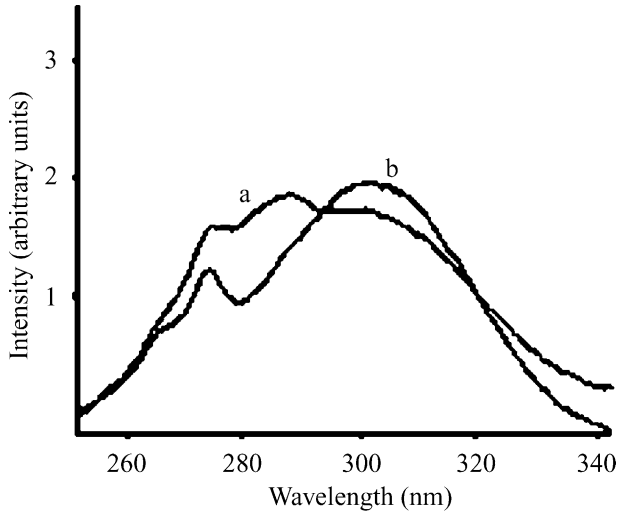

Fig. 3. Excitation spectra of ϵ-ATP (acceptor), in the presence and absence of Pimpernel apyrase (donor). Excitation spectrum (followed at λ_{em} = 413 nm) of 1.1 μM ε-ATP in 100 mM MES, pH 6.0, alone (curve b) or in the presence of 3.3 μM Pimpernel apyrase (curve a). The intensity values of these excitation spectra were normalised at λ_{ex} 325 nm.

between their absorption spectra and the Trp emission, is also accompanied by quenching of the protein Trp. Therefore, probably in their quenching effect of Trp emission, in addition to the conformational changes deduced from the effect of non-fluorescent nucleotide analogues, the phenomenon of energy transfer from Trp residues to their fluorescent portions is also involved. Due to secondary dispersion of the lamp beam, it was not possible to determine energy transfer using this method with TNP-derivatives. With MANT-derivatives a strong absorption at 340 nm masked the differences in emission centred around 280 nm.

2.4. Dissociation constants for the complexes of Pimpernel and Desirée apyrases with nucleotide analogues

Fluorescent spectroscopy is a suitable method to measure ligand binding to proteins by following changes in the intrinsic protein fluorescence upon binding of the ligand (Lakowitz, 1983). Titration data of Pimpernel apyrase obtained with the non-hydrolysable analogues, ADP-PCP or AMP-PCP in the presence or absence of calcium ions, fitted well to hyperbolic curves (not shown). Similar titration data were also obtained with MANT-, TNP-, and ε-nucleotides in the absence of calcium ions to avoid their hydrolysis. Dissociation constants (K_d) of all Pimpernel nucleotide complexes are summarized in Table 1. For comparison purposes, we have included in the table data previously reported by our group for the Desirée enzyme (Espinosa et al., 2000). It can be seen that, in the case of the Pimpernel apyrase, K_d values for the triphosphorylated analogues are 2- to 8-fold lower than the corresponding values for diphosphorylated compounds. These results differ with the data obtained for the Desirée apyrase.

Table 1
Dissociation constants (K_d) of different nucleotide derivatives bound to apyrase in the absence of Ca^{2+}

Nucleotide	K_d (μM^{-1})±S.D.	
	Pimpernel[a]	Desirée[b]
ADP-PCP	2.1±0.7	16±9
AMP-PCP	17±8	32±5
MANT-ATP	23±12	257±78
MANT-ADP	45±17	139±48
TNP-ATP	8±5	249±46
TNP-ADP	29±18	197±63
ε-ATP	116±53	10±5
ε-ADP	234±104	3±2

[a] Calculations were based on the decrease in the fluorescence intensity at the maximum wavelength of emission at 340 nm. The nucleotide range used was 0–500 μM. Data are means of at least five independent titration experiments with their corresponding standard deviation (S.D.).
[b] Data from Espinosa et al. (2000).

These differences in binding affinities for di- and triphosphorylated substrates may be related to the higher ATPase/ADPase ratio of the Pimpernel enzyme. Other differences between Desireé and Pimpernel apyrases are the higher affinity of the Desirée apyrase for ϵ-derivatives and the higher affinity of Pimpernel for TNP- and MANT- nucleotides. These last observations suggest specific binding constraints for different regions of the nucleotide in the two apyrases: substitution in the ribose hydroxyls of the nucleotide lowers the binding affinity to the Desirée enzyme, while substitution in the nucleotide base lowers the binding affinity to the Pimpernel enzyme.

3. Concluding remarks

Both Pimpernel and Desirée enzymes exhibit energy transfer between Trp residues and ϵ-nucleotides supporting the proposal that at least some Trp are in or near the nucleotide-binding region. Although Pimpernel and Desirée apyrases contain four Trp each, quenching experiments indicate differences between both enzymes in their location within the tertiary structure. This is particularly evidenced by ionic quenchers (I^-, Cs^+), which detect the presence of residues inaccessible to these quenchers in the Pimpernel apyrase. Nucleotide binding alters both the fluorescence spectra of these enzymes and the accessibility of Trp residues to quenchers, suggesting conformational changes in the apyrase upon nucleotide binding. Pimpernel apyrase differs from Desirée in a higher affinity for triphosphorylated analogues than for the diphosphorylated ones. Data also show that substitutions in the ribose hydroxyls or in the adenine base have different consequences in the binding affinity of the ligand to the two apyrases, suggesting different subtrate binding constraints in the active site of these enzymes. The amino acid analysis of both isoenzymes indicated differences in their sequences. The present subject of study of our laboratory is to obtain information on apyrase sequence using the approach reported by Handa and Guidotti (1996) by cloning the cDNA for the potato apyrase from these two varieties. This would be valuable information for understanding the kinetic and fluorescence differences between the isoenzymes. In addition, by means of modeling studies, the sequences would generate additional knowledge about possible differences in their three-dimensional structures.

4. Experimental

4.1. Materials

TNP-ATP, TNP-ADP, ϵ-ATP, ϵ-ADP were obtained from Molecular Probes Inc. MANT-ATP and MANT-ADP were synthesized and purified as previously described (Hiratzuka, 1983).

4.2. Apyrase purification

Apyrase was purified from homogeneous strains of *S. tuberosum* cv Pimpernel and Desirée as previously reported (Kettlun et al., 1982; Espinosa et al., 2000). The homogeneity of this enzyme was checked both by gel isoelectrofocusing and SDS-PAGE (Kettlun et al., 1982). Protein concentrations were determined as in Lowry et al. (1951) with bovine serum albumin (BSA) as standard.

4.3. Determination of Trp number in Pimpernel apyrase

Pure Pimpernel apyrase was exhaustively dialysed against 0.1 M $NaHCO_3$ and further freeze dried. Hydrolysis was carried out under normal and thioglycolic acid conditions (Matsubara and Sasaki, 1969). Thiglycolic acid was used as a Trp protector. Amino acids were analysed on a Beckman 6300 ion-exchange amino acid analyser with external calibration. A parallel run with BSA was used to standarize the determination.

4.4. Spectroscopic measurements

Titrations with substrate analogues (ADP-PCP and AMP-PCP) and fluorescent nucleotide analogues (MANT-ATP, MANT-ADP, TNP-ATP, TNP-ADP, ε-ATP and ε-ADP) were performed using 0.06 mg/ml of protein (1.2 μM). The medium contained 100 mM MES pH 6.0, in the presence of appropriate EDTA concentration for chelation of contaminant calcium. Measurements were at 20 °C, with a SPEX FL2-Z2 spectrofluorometer and a 0.5-cm quartz semimicro cuvette.

The absorption spectra of apyrases showed a maximum at 286 nm. The absence of a shoulder in the emission at 308–310 nm implies that the emission observed arises mainly from Trp residues without important contribution of tyrosyl residues (Encinas et al., 1993). The binding of the nucleotide analogues was followed by monitoring changes of the protein Trp fluorescence emission ($\lambda_{exc} = 286$ nm; $\lambda_{em} = 340$ nm). Nucleotide analogues were freshly prepared in MES 100 mM pH 6.0 and their concentrations were calculated from their absorption maxima (Espinosa et al., 2000). Fluorescence was measured as described and corrected by subtracting the emission spectrum of the buffer used in the last purification step, containing identical amounts of fluorescent analogues in the absence of apyrase. Protein fluorescence intensity and absorbance were measured, and appropriate corrections were made for dilution effects (never exceeding 10%). Corrections to account for the inner filter effect were made (Espinosa et al., 2000).

4.5. Estimation of fluorescence energy transfer from apyrase tryptophans to ϵ-nucleotides bound to the active site

Following Bujalowski and Klonowska (1994b), the fluorescent intensity of the acceptor (in our case ε-ATP and ε-ADP) excited at the maximum wavelength of the donor (286 nm in the case of Trp apyrases) was measured in the presence and absence of the protein. An increase in intensity in the excitation spectrum of the acceptor, in the presence of the protein, centred around the donor wavelength (286 nm) would be the result of an efficient energy transfer from the Trp to the nucleotide. The enzyme and nucleotide concentrations were 3.3×10^{-6} M and 1.1×10^{-6} M, respectively.

4.6. Titration data analysis

Data from the titration of the enzyme with the different nucleotide analogues were fitted to hyperbolic curves using the program Microcal Origin 4.1.

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