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Fluorescence studies of ATP-diphosphohydrolase from *Solanum tuberosum* var. Desirée

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Abstract

Chemical modification of potato apyrase suggests that tryptophan residues are close to the nucleotide binding site. K_d values ($\pm$ Ca^{2+}) for the complexes of apyrase with the non-hydrolysable phosphonate adenine nucleotide analogues, adenosine 5′-(β,γ-methylene) triphosphate and adenosine 5′-(α,β-methylene) diphosphate, were obtained from quenching of the intrinsic enzyme fluorescence. Other fluorescent nucleotide analogues (2′(3′)-O-(2,4,6-trinitrophenyl) adenosine 5′-triphosphate, 2′(3′)-O-(2,4,6-trinitrophenyl) adenosine 5′-diphosphate, 1,N^6-ethenoadenosine triphosphate and 1,N^6-ethenoadenosine diphosphate) were hydrolysed by apyrase in the presence of Ca^{2+}, indicating binding to the active site. The dissociation constants for the binding of these analogues were calculated from both the decrease of the protein (tryptophan) fluorescence and enhancement of the nucleotide fluorescence. Using the sensitised acceptor (nucleotide analogue) fluorescence method, energy transfer was observed between enzyme tryptophans and ethene-derivatives. These results support the view that tryptophan residues are present in the nucleotide-binding region of the protein, appropriately oriented to allow the energy transfer process to occur.

Keywords: *Solanum tuberosum*; Potato; Solanaceae; Intrinsic and extrinsic fluorescence; ATP-diphosphohydrolase; Apyrase

1. Introduction

Apyrase (ATP-diphosphohydrolase, E.C. 3.6.1.5) catalyses hydrolysis of pyrophosphate bonds in nucleoside di- and triphosphates in the presence of a divalent metal ion with sequential release of two equivalents of inorganic phosphate (Traverso-Cori et al., 1965). This enzyme has been described in both animal and plant tissues (Komoszynski and Wojtczak, 1996). In animal tissues, apyrase is an ectoenzyme whose function has been related to regulation of extracellular nucleotide concentrations (Espinosa et al., 1996; Plesner, 1995). In contrast, the enzyme from plant tissues, such as potato, is predominantly soluble (Valenzuela et al., 1989; Kettlun et al., 1992a, 1992b). Physiologically, potato apyrase probably removes ADP and other nucleoside diphosphates, released as by-products during the biosynthesis of starch and cell walls (Anich et al., 1990).

The true substrate of apyrase is probably the nucleotide-divalent metal ion complex (Valenzuela et al., 1988). Apyrases isolated from different clonal varieties of *S. tuberosum* differ in their relative rates of hydrolysis of ATP and ADP (Kettlun et al., 1992a, 1992b, 1982; Traverso-Cori et al., 1970). In particular, the enzyme from the var. Desirée catalyses hydrolysis of the γ-phosphoryl group of ATP at the same rate as that of the β-phosphoryl group of ADP (Kettlun et al., 1982). Chemical modification studies of the Desirée enzyme implicate the involvement of His, Arg, and Tyr, carboxylic acid groups and Trp amino acid resi-

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dues located in the neighbourhood of the nucleotide-binding region (Kettlun et al., 1982).

Tryptophan fluorescence is a useful tool for studying protein structure, function, and dynamics (Encinas et al., 1993; Bujalowski and Klonowska, 1994a, 1994b). Here we describe intrinsic and extrinsic fluorescence studies with Desirée apyrase, using fluorescent and non-fluorescent nucleotide analogues. We provide evidence of substrate-induced conformational changes, report reliable calculated values for dissociation constants for several enzyme-nucleotide complexes, and demonstrate fluorescence energy transfer from protein Trp residues to adenine-substituted nucleotide analogue acceptors.

2. Results and discussion

2.1. Hydrolytic activity of Desirée apyrase on different fluorescent nucleotide derivatives

The ribose-modified fluorescent analogues MANT-ATP, MANT-ADP, TNP-ATP and TNP-ADP, and the base-modified fluorescent analogues ε-ATP and ε-ADP were all hydrolysed by apyrase in the presence of Ca^{2+}. The relative activities of the enzyme with these nucleotides in comparison with those for the physiological substrates ATP and ADP are shown in Table 1. Modifications in the base did not significantly affect the relative hydrolysis rate (ε-ATP, ε-ADP), while the activities with ribose-substituted analogues (MANT- and TNP-derivatives) were somewhat lower than with the physiological substrates. Lower ATPase/ADPase activity ratios were also found with the latter analogues. These data agree with those of previous experiments which showed that potato apyrase has a rather low specificity for the non-phosphate part of the molecule (Del Campo et al., 1977), even though changes in the pyrophosphate chain resulted in significant effects (Kettlun et al., 1982; Del Campo et al., 1977).

Table 1
Comparative hydrolytic activity of apyrase on adenine nucleotides and fluorescent derivatives[a]

Nucleotide	V (μmol P_i/min × ml) ± SD	ATPase/ADPase ratio
ATP	74.2 ± 12.1	0.97
ADP	76.8 ± 9.2	
ε-ATP	72.3 ± 8.5	0.98
ε-ADP	73.9 ± 10.2	
TNP-ATP	33.6 ± 3.5	2.87
TNP-ADP	11.7 ± 1.8	
MANT-ATP	52.8 ± 4.1	1.17
MANT-ADP	45.3 ± 5.2	

[a] The nucleotide concentration was 0.4 mM in 100 mM MES pH 6.0, in the presence of 1 mM $CaCl_2$. The data are means of two independent measurements ± SD.

2.2. Quantification of the number of tryptophan residues

We found 4–5 residues (4.17 ± 0.19) after hydrolysis under normal and thioglycollic acid conditions (Matsubara and Sasaki, 1969). This value is twice that obtained previously by the spectrophotometric method of Trp oxidation with *N*-bromosuccinimide (Mancilla et al., 1984). The value reported here is likely to be more accurate because of the higher sensitivity of the method used. This higher value is also closer to the figure of five deduced from analysis of commercial potato apyrase cDNA (Handa and Guidotti, 1996).

2.3. Intrinsic fluorescence studies

Apyrase exhibits a fluorescence spectrum character-

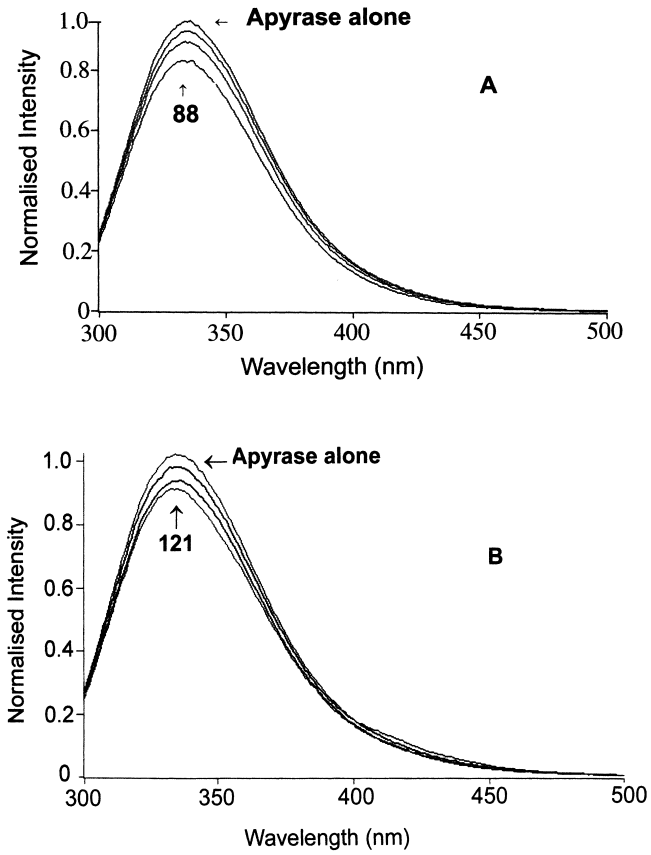

Fig. 1. Effect of ATP and ADP phosphonates on the fluorescence emission spectra of apyrase. Apyrase concentration in 100 mM MES pH 6.0 was 1.2 μM (0.06 mg/ml). The excitation wavelength was 286 nm. The spectra were corrected by substraction of the spectra of a mixture of equal amounts of nucleotide but without enzyme. The values of fluorescence emission intensity were normalised by assigning 1 to the higher value of the protein emission alone. (A) Titration of apyrase with 0–88 μM of ADP-PCP; (B) titration of apyrase with 0–121 μM of AMP-PCP.

istic of moderately buried tryptophan residues (Burstein et al., 1973; Athes et al., 1998) with a maximum at 337 nm and a half-band width of 50 nm. The absence of a shoulder at 308–310 nm implies that the fluorescence emission arises mainly from the Trp residues (Encinas et al., 1993).

Addition of increasing amounts of non-hydrolysable ATP and ADP phosphonates in the presence of Ca^{2+} decreases the Trp fluorescence intensity, but without an appreciable shift in the emission maximum at 337 nm. Fig. 1 shows, as an example, the spectral changes with ADP-PCP–Ca^{2+} and AMP-PCP–Ca^{2+}; the maximum quenching effects were 31.6 and 19.2%, respectively (Table 2). When free nucleotides were used, minor quenching effects (17.6–22.7%) were observed (Table 2). These changes in fluorescence intensity indicate that the local environment of Trp residues is altered by nucleotide binding. The quenching of Trp fluorescence by amino acid side chains and the effect of proximity, specific geometry, and polarity on the quenching efficiency of particular quenchers has been discussed recently (Chen and Barkley, 1998).

The fluorescence changes produced by binding of ligands to proteins can be used to determine dissociation constants (Encinas et al., 1993; Guixé et al., 1998). All our data fitted well to hyperbolic curves, and Fig. 2(A) shows the intrinsic fluorescence changes of apyrase as a function of the concentrations of the phosphonate derivative in the absence of Ca^{2+}. K_d values, calculated for ATP phosphonate and ADP phosphonate both with and without Ca^{2+}, are summarised in Table 2. K_d values determined for phosphonate analogues are similar to K_i values for these compounds as competitive inhibitors for ATP and ADP (0.02–0.07 mM) (Kettlun et al., 1982). The presence of Ca^{2+} made little difference in the measured K_d values.

The diphosphorylated nucleoside produced a larger decrease in Trp fluorescence; this could imply differential ligand-induced conformational changes. These changes, however, do not expose the Trp residues to less hydrophobic environments, because no change in the maximum emission wavelength was observed. The decrease can thus be attributed to movements in amino acid residues in the neighbourhood of Trp, and these could be responsible for the quenching effects (Guixé et al., 1998; O'Donoghue et al., 1992).

2.4. Extrinsic fluorescence studies

Based on chemical modification studies, the proposal has been put forward that Trp residues could be implicated in the nucleotide binding region of potato apyrase (Kettlun et al., 1992b, 1982). According to this proposal, the binding of appropriate fluorescent nucleotide analogues to apyrase should lead to energy transfer from Trp to the fluorescent moiety, provided that the energy donors are located at distances and with orientations suitable for the energy-transfer process to take place (O'Donoghue et al., 1992). Because of potential hydrolysis of the different ATP and ADP derivatives by apyrase, fluorescence studies with these compounds were carried out without Ca^{2+}, conditions under which hydrolysis does not occur. For these experiments, the required amount of EDTA was added to chelate calcium introduced with nucleotides. The maximum emission intensities of MANT-, TNP- and ethene derivatives of ATP and ADP were all enhanced by apyrase without detectable spectral shift (not shown). The increase in fluorescence can be attributed to the binding of the fluorescent portion of the nucleotides to a hydrophobic region of the protein, because decreasing solvent polarity increases the quantum yield of these fluorophores (Bujalowski and Klonowska, 1994b; Faller, 1990; Cremo et al., 1990). Fig. 2(B) and (C) show, as examples, the hyperbolic fits of the titration data with TNP-ATP and MANT-ATP, respectively.

The large emission values of ethene- and MANT-derivatives masked the enhancement of tryptophan emission intensities. Therefore, the high fluorescence prevented us from using the appropriate concentration range. Instead, K_d values were calculated from the quenching effect of these compounds on protein Trp fluorescence. Only when TNP-analogues were used we could determine the dissociation constant by following the changes in emission at both 337 and 555 nm. Estimated dissociation constants

Table 2
Values of K_d for the binding of different fluorescent nucleotide derivatives with and without Ca^{2+} [a]

Nucleotide	K_d (μM^{-1}) ± SD	Maximum emission (nm)	Maximum decrease in fluorescence emission observed (%)
ADP-PCP	14 ± 6	335	22.7
AMP-PCP	29 ± 1	336	17.6
ADP-PCP + Ca^{2+}	5 ± 1	333	31.6
AMP-PCP + Ca^{2+}	26 ± 15	335	19.2

[a] Calculations were based on the decrease in the fluorescence intensity at the maximum wavelength of emission of Trp. The nucleotide range used was 0–200 μM for ADP-PCP and AMP-PCP. Data are means of at least three independent experiments.

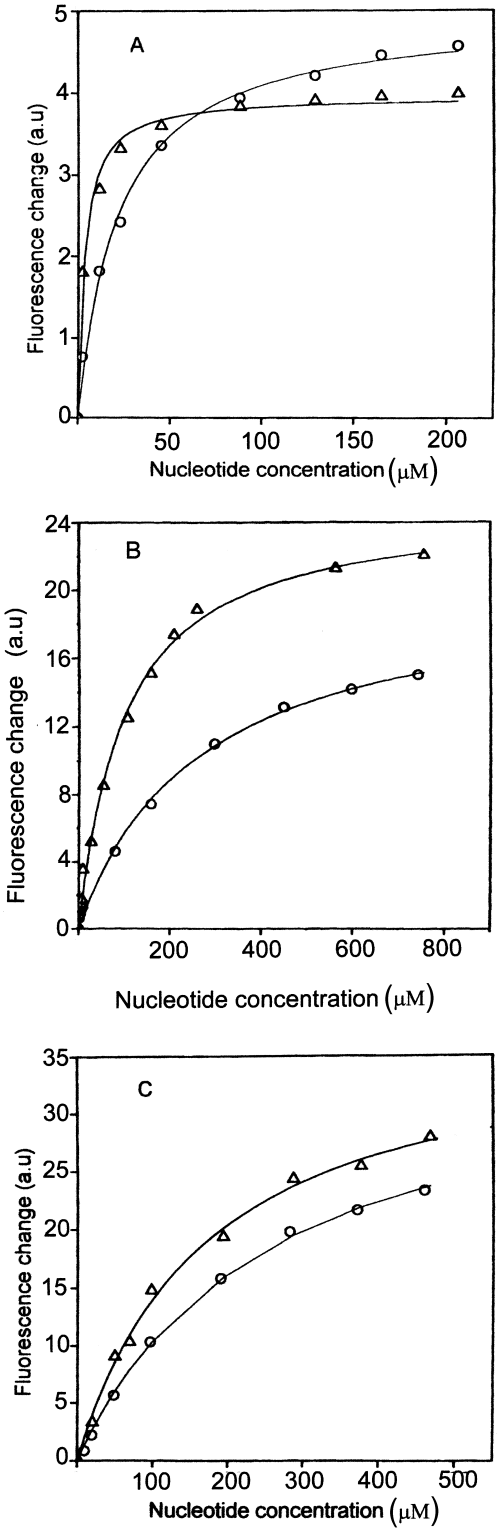

Fig. 2. Intrinsic fluorescence changes of Desirée apyrase as a function of nucleotide concentration. Fluorescence change for apyrase (1.2 μM, 0.06 mg/ml) is plotted as a function of: (A) ADP-PCP (○), and AMP-PCP (△), in the absence of Ca^{2+}; (B) MANT-ATP (○), and MANT-ADP (△), in the absence of Ca^{2+}; (C) TNP-ATP (○), and TNP-ADP (△), in the absence of Ca^{2+}.

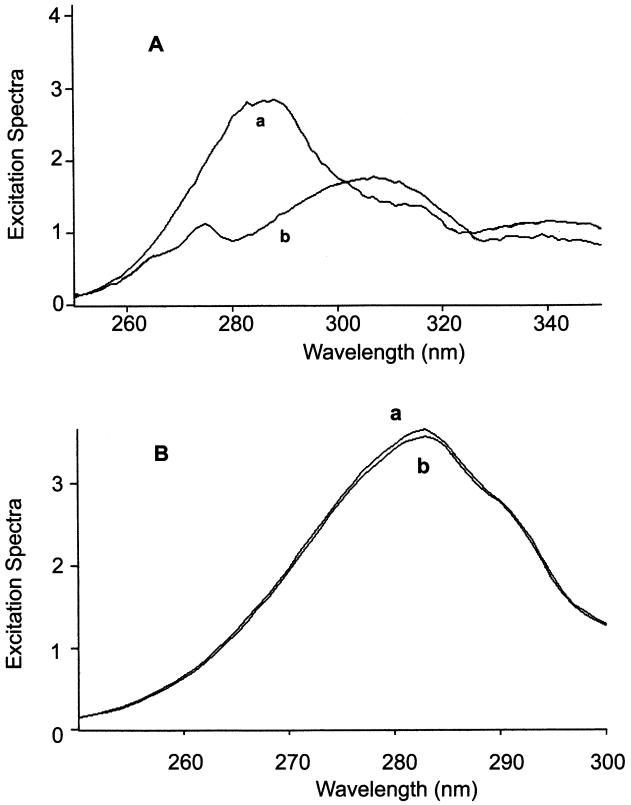

Fig. 3. Excitation spectra of the acceptor, in the presence of the donor. (A) Excitation spectrum ($\lambda_{em} = 405$ nm) of 1.1 μM ethene-ADP in 100 mM MES, pH 6.0, in the presence of 3.3 μM Desirée apyrase (a) and alone (b). (B) Superimposition of differential excitation spectra: ($\lambda_{em} = 337$ nm) of 1.1 μM ethene-ADP in presence or absence of 3.3 μM Desirée apyrase (a) and the excitation spectrum of 3.3 μM Desirée apyrase (b). The intensity values of these excitation spectra were normalised at 325 nm.

for the different fluorescent nucleotides in the calcium-free form are summarised in Table 3. K_d values for TNP-derivatives with modification in the ribose moiety are one order of magnitude larger than the K_m values determined for ATP–Ca and ADP–Ca (25 and 100 μM, respectively) (Kettlun et al., 1982). For the ethene-derivatives, K_d values are of the same magnitude. Fluorescent nucleotides quench Trp fluorescence much more than do non-fluorescent derivatives. This could be because of either different conformational changes produced by the presence of bulky groups on the ligands or the occurrence of energy transfer phenomena.

TNP-nucleotides are only weakly fluorescent in aqueous solutions. The quantum yield of TNP-nucleotide fluorescence can, however, be significantly enhanced when these nucleotides are bound in the hydrophobic environment of proteins (Bujalowski and Klonowska, 1994a; Hiratzuka, 1985; Cheng and Koland, 1996). Although no shift in the emission maximum was observed, the large enhancement in the fluorescence

emission of TNP-ATP and TNP-ADP when the nucleotides were added to apyrase (50.9 and 65.4 fold, respectively), suggests that the TNP moiety of the analogue is relatively buried or rigidly positioned in the TNP-nucleotide-apyrase complex. The binding of MANT-, TNP-, and ethene-derivatives accompanied by quenching of the protein Trp could be because of either fluorescence energy transfer from the Trp to the nucleotide or to conformational changes (Bujalowski and Klonowska, 1994a, 1994b; Lakowitz, 1983). The first possibility requires that the acceptor absorption spectrum significantly overlaps with the protein fluorescence spectrum. The maximum wavelength of excitation of MANT- and ethene-derivatives is between 325 and 340 nm, and for TNP-derivatives this maximum value is slightly higher (405 nm) (Bujalowski and Klonowska, 1994b). Because in the three cases there is a significant spectral overlap, energy transfer should occur if the Trp residues are in close proximity and correctly oriented to the bound nucleotide. To test this point we used the sensitised acceptor (nucleotide analogue) fluorescence approach described in (Bujalowski and Klonowska, 1994b); this is a reliable method for examining this phenomenon. The excitation spectra ($\lambda_{em} = 413$ nm) of ε-ADP with and without enzyme are shown in Fig. 3(A). The spectra have been normalised at $\lambda_{ex} = 325$ nm, where there is no excitation of the protein tryptophans and the change in nucleotide fluorescence is because of the changes in their quantum yield produced by binding to the enzyme (Bujalowski and Klonowska, 1994b). The excitation spectrum of ε-ADP in the presence of apyrase shows an increase centred at 283 nm compared to the excitation spectra of the nucleotide analogue alone. The difference between the two excitation spectra of ε-ADP (recorded with and without protein) coincides with the excitation spectrum of the enzyme (Fig. 3(B)). In the spectral range 250–300 nm, the ethene-derivatives do not absorb in the concentration range used. Similar data were obtained with ε-ATP (data not shown). From these results, we conclude that the increase in the ethene-nucleotide fluorescence in the presence of the protein is a result of an efficient energy transfer from some of the Trp residues to the ethene-nucleotide analogues.

Due to secondary dispersion of the lamp beam, it was not possible to determine energy transfer using this sensitised method with TNP-derivatives. In contrast, the MANT-derivatives have a strong absorption at 340 nm, which masked the differences in emission centred around 280 nm.

In conclusion, these results show protein conformational changes when free and Ca^{2+}-complexed nucleotide analogues are bound to Desirée apyrase. The demonstration of energy transfer between some of the 4–5 Trp residues and ε-ADP and ε-ATP, supports the proposal that Trp residues are close to the nucleotide-binding region (Kettlun et al., 1992b, 1982), and imply that they are appropriately oriented for the phenomenon to occur.

3. Experimental

3.1. Materials

TNP-ATP, TNP-ADP, ε-ATP, ε-ADP and methylisatoic anhydride were obtained from Molecular Probes Inc. ATP, ADP, Sepharose 4B-Cl and Cibacron Blue were purchased from Sigma (Saint Louis, MO). MES and ammonium sulfate were obtained from J.T. Baker.

Table 3
Dissociation constants of the fluorescent analogues[a]

Nucleotide	Concentration range used (M)	K_d (μM^{-1}) ± SD	Wavelength (nm)	Maximum change in fluorescence emission
MANT-ATP	0–742	260 ± 80	337	95%[b]
MANT-ADP	0–259	140 ± 50	337	78%[b]
TNP-ATP	0–460	250 ± 50	337	77%[b]
TNP-ATP	0–460	230 ± 60	555	51 fold[c]
TNP-ADP	0–468	200 ± 60	337	70%[b]
TNP-ADP	0–468	250 ± 80	555	65 fold[c]
ε-ATP	0–177	10 ± 5	337	49%[b]
ε-ADP	0–161	3 ± 2	337	41%[b]

[a] Experiments were performed in the presence of EDTA at a concentration calculated in each case according to the contaminant calcium ion determined by atomic absorption spectrometry. Measurements were performed either following the Trp emission (λ_{em} 337 nm) or, as in the case of TNP-derivatives, from the maximum emission of the TNP-group (λ_{em} 555 nm). Data are means of at least three independent experiments.

[b] Quenching effect on fluorescence intensity determined at the highest nucleotide concentration used, expressed as the percentage of decrease of the fluorescence emission of the protein alone.

[c] Amount of increase in fluorescence emission of the fluorescent portion of the TNP-derivatives determined at the highest nucleotide concentration used. For each titration point the fluorescence emission of the unbound nucleotide was substracted.

3.2. Apyrase purification

Apyrase was purified from a homogeneous strain of *S. tuberosum* cv Desirée, obtained by clonal selection, and generously supplied by the Instituto de Investigaciones Agropecuarias "Remehue", Osorno, Chile. The enzyme was prepared as previously reported (Kettlun et al., 1982) except for the last step where the affinity chromatography column had between Sepharose Cibacron blue and Sepharose 4B-Cl in the proportion of 1 : 1. Elution of the enzyme was achieved with a gradient of 0–2 M NaCl. Homogeneity of this preparation was checked both by gel isoelectrofocusing (Kettlun et al., 1982) and SDS-PAGE (Laemmli, 1970).

3.3. Apyrase activity

Release of inorganic phosphate from ATP, ADP, and the fluorescent analogues was measured as described (Chen et al., 1956). The enzymatic assay, at 30°C, employed 0.1 M sodium succinate pH 6.0 and 5 mM $CaCl_2$ (Traverso-Cori et al., 1965). The substrate concentration (ATP and ADP) during the purification steps was 2 mM in 0.1 M sodium succinate pH 6.0 and 5 mM $CaCl_2$, and 0.4 mM on 100 mM MES pH 6.0 and 1 mM $CaCl_2$ for the comparison of the activities of the fluorescent analogues.

3.4. Protein determination

During purification, protein concentrations were determined by absorption at 280 nm. The extinction coefficient of the pure enzyme is 9.01×10^4 M^{-1} cm^{-1}. With homogeneous apyrase, the method described (Lowry et al., 1951) was used with BSA as standard.

3.5. Determination of the amount of calcium present in the medium by spectroscopic measurements

Total calcium ion concentration was determined by atomic absorption spectrometry.

3.6. Determination of the number of Trp of apyrase

Pure apyrase samples were exhaustively dialysed against 0.1 M $NaHCO_3$ and further freeze-dried. After hydrolysis under normal and thioglycollic acid conditions, amino acids were identified and quantified on a Beckman 6300 ion-exchange amino acid analyser with external calibration (Matsubara and Sasaki, 1969).

3.7. Spectroscopic measurements

Titration with substrate analogues (ATP and ADP phosphonates) and fluorescent nucleotide analogues (MANT-ADP-PCP, MANT-AMP-PCP, TNP-ATP, TNP-ADP, ε-ATP and ε-ADP) was performed with 0.06 mg/ml of protein (1.2 μM). The medium contained 100 mM MES pH 6.0, in the presence of $CaCl_2$ (2.5 times the nucleotide concentration) or the appropriate EDTA concentration for chelation of added calcium. Measurements were at 20°C, with a SPEX FL2-Z2 spectrofluorometer and a 0.5 cm quartz semi-microcuvette.

The binding of the nucleotide analogues was followed by monitoring either changes of the protein Trp fluorescence emission ($\lambda_{ex} = 286$ nm; $\lambda_{em} = 335$–337 nm) and the increase in fluorescence emission of the fluorescent analogues. Conditions were: MANT-ATP, MANT-ADP ($\lambda_{ex} = 340$ nm; $\lambda_{em} = 447$ nm); TNP-ATP, TNP-ADP ($\lambda_{ex} = 406$ nm; $\lambda_{em} = 555$ nm) and ε-ATP, ε-ADP ($\lambda_{ex} = 325$ nm; $\lambda_{em} = 413$ nm). Nucleotide analogues were freshly prepared in 100 mM MES pH 6.0 and their concentrations were calculated from their absorption maxima. Molar extinction coefficients are: ATP phosphonate (A_{259} nm: 14,500 M^{-1} cm^{-1}), ADP phosphonate (A_{259} nm: 15,400 M^{-1} cm^{-1}); MANT-nucleotides (A_{356} nm: 5800 M^{-1} cm^{-1}); TNP-derivatives (A_{408} nm: 26,400 M^{-1} cm^{-1}); ethene-derivatives (A_{275} nm: 5600 M^{-1} cm^{-1}).

Fluorescence was measured as described and corrected by subtracting the emission spectrum of the column buffer containing identical amounts of buffer and different concentration of fluorescent analogue in the absence of protein. 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 according to the relationship $I = Iobs\ 10^{A/2}$, where A is the increase in absorbance at the centre of the cuvette (Encinas et al., 1993).

3.8. Estimation of the efficiency of the fluorescence energy transfer from the apyrase tryptophans to the nucleotide analogue bound in the nucleotide-binding site

Briefly this method (described in Bujalowski and Klonowska, 1994b) consists of measuring the fluorescence intensity of the acceptor (ε-ATP or ε-ADP) excited at 286 nm (Trp excitation) and in the absorption band of the analogue with and without apyrase. The enzyme concentration was 3.3×10^{-6} M and the nucleotide concentration was 1.1×10^{-6} M. An increase in the excitation spectrum of acceptor, in the presence of protein, centred around the wavelength of tryptophan absorption (286 nm), would be a result of an efficient energy transfer from the Trp to the nucleotide. On the other hand, there should be a superposition of the difference between the excitation spectrum of the analogue in the presence of protein and analogue alone, and the excitation spectrum of protein (at

the corresponding maximum emission wavelength of each derivative).

3.9. Synthesis of nucleotide analogues

MANT-ATP and MANT-ADP were synthesised and purified as described previously (Hiratzuka and Uchida, 1983).

3.10. 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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Intrinsic and extrinsic fluorescence studies of this enzyme, which split pyrophosphoric bonds of ATP or ADP, provide evidence of substrate-induced conformational changes and fluorescence energy transfer from Trp residues to fluorescent-substituted nucleotides.

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