Studies on ATP-diphosphohydrolase nucleotide-binding sites by intrinsic fluorescence

A.M. Kettlun, V. Espinosa, A. Zanocco and M.A. Valenzuela

Departamento de Bioquímica y Biologia Molecular, Universidade de Chile, Santiago, Chile

Abstract

Correspondence

A.M. Kettlun
Departamento de Bioquímica y
Biologia Molecular
Universidade de Chile
Casilla 174, Correo 22
Santiago
Chile

Potato apyrase, a soluble ATP-diphosphohydrolase, was purified to homogeneity from several clonal varieties of *Solanum tuberosum.* Depending on the source of the enzyme, differences in kinetic and physicochemical properties have been described, which cannot be explained by the amino acid residues present in the active site. In order to understand the different kinetic behavior of the Pimpernel (ATPase/ADPase = 10) and Desirée (ATPase/ADPase = 1) isoenzymes, the nucleotide-binding site of these apyrases was explored using the intrinsic fluorescence of tryptophan. The intrinsic fluorescence of the two apyrases was slightly different. The maximum emission wavelengths of the Desirée and Pimpernel enzymes were 336 and 340 nm, respectively, suggesting small differences in the microenvironment of Trp residues. The Pimpernel enzyme emitted more fluorescence than the Desirée apyrase at the same concentration although both enzymes have the same number of Trp residues. The binding of the nonhydrolyzable substrate analogs decreased the fluorescence emission of both apyrases, indicating the presence of conformational changes in the neighborhood of Trp residues. Experiments with quenchers of different polarities, such as acrylamide, Cs^+ and I^- indicated the existence of differences in the nucleotide-binding site, as further shown by quenching experiments in the presence of nonhydrolyzable substrate analogs. Differences in the nucleotide-binding site may explain, at least in part, the kinetic differences of the Pimpernel and Desirée isoapyrases.

Introduction

Apyrase (ATP-diphosphohydrolase, EC 3.6.1.5) has been described both as a soluble enzyme in plants (1) and as a membrane-bound protein in animal tissues (2,3) and parasites (4). Apyrase catalyzes the hydrolysis of pyrophosphate bonds of nucleosides di- and triphosphate in the presence of bivalent metal ions (5). The best effect is obtained in the presence of Ca^{2+}, although other ions such as Mn^{2+}, Co^{2+}, Mg^{2+} and Zn^{2+} also stimulate the enzymatic activity (6). We purified apyrase from various clonal varieties of potato tubers and we found that the ATPase/ADPase ratio depends on the clonal variety used as a source

of the enzyme (7). Because of their extreme ATPase/ADPase ratio, the Pimpernel and Desirée varieties of *Solanum tuberosum* were selected for the present study.

Pimpernel apyrase hydrolyzes ATP ten times faster than ADP, while the Desirée enzyme splits both nucleotides at the same rate. We purified and characterized both isoapyrases and found that they differ in pI. Although the sum of the acid amino acid residues and the total number of basic residues is identical for the two isoenzymes, Pimpernel apyrase is two pH units more alkaline than Desirée apyrase (8). The enzymes also differ in the optimum pH of ADPase activity (5) and are very similar in molecular mass, approximately 49 kDa (6), in amino acid composition (8) and in the residues involved in the catalytic activity studied by chemical modification. This approach has permitted us to propose that Arg, Tyr, COO⁻ and Trp residues may be involved in apyrase activities (9), while SH groups are unimportant for the catalytic activities of both apyrases (6). Since Koshland reagent inactivated both isoapyrases, and substrates protected them from inactivation, tryptophyl residues are probably involved in the nucleotide-binding site (6). Intrinsic fluorescence experiments on the Desirée enzyme (10) also suggest the presence of Trp near the nucleotide-binding site.

In order to investigate the environment of these residues in both isoapyrases, we studied the quenching of the Trp fluorescence of Pimpernel and Desirée isoenzymes using quenchers of different polarities.

Material and Methods

ATP, ADP, the nonhydrolyzable substrate analogs ATP and ADP phosphonates (ADP-PCP and AMP-PCP), Sepharose 4B-Cl and Cibacron blue were purchased from Sigma Co. (St. Louis, MO, USA). MES and ammonium sulfate were obtained from J.T. Baker (Phillipsburg, NJ, USA).

Apyrase purification

The apyrases were purified from homogeneous strains of *S. tuberosum* cv Pimpernel and Desirée obtained by clonal selection, and generously supplied by the Instituto de Investigaciones Agropecuarias "Remehue", Osorno, Chile. The enzymes were prepared as previously reported (10). The homogeneity of the proteins was checked both by gel isoelectrofocusing (6) and SDS-PAGE (11).

Protein determination

Protein concentration was determined by UV absorption at 280 nm.

Spectroscopic measurements

The emission spectra were determined with a SPEX FL2-Z2 spectrofluorometer and a 0.5-cm quartz semi-microcuvette. The excitation wavelength was 286 nm and measurements were performed at 20ºC. Protein concentrations were 0.071 mg/ml for the Desirée enzyme and 0.064 mg/ml for the Pimpernel apyrase.

Quenching studies

An aliquot of 0.06 mg/ml of each apyrase was titrated in its native conformation in 100 mM MES, pH 6.0, or denatured with 4.8 M guanidine hydrochloride (GndHCl) in 100 mM MES, pH 7.4. The intrinsic fluorescence of the proteins was quenched with 5-M solutions of acrylamide, cesium chloride, and sodium iodide prepared in 100 mM MES, pH 6.0. Titration was performed using a concentration range of quenchers from 0 to 0.35 M. Protein titration with sodium iodide was performed in the presence of 5 M sodium chloride to keep the ionic strength constant, and in 0.1% $Na_2S_2O_3$ to avoid I_3^- formation in 100 mM MES.

Results

Intrinsic fluorescence of Pimpernel and Desirée apyrases

The two enzymes differed slightly in the maximum emission wavelength, which was 340 nm for Pimpernel and 336 nm for Desirée. The effect of ADP phosphonate on the fluorescence spectra of both isoenzymes is shown in Figure 1. The addition of the non-hydrolyzable ADP derivative produced a considerable decrease in the Trp fluorescence intensity of both isoapyrases. The results with the ATP derivative were very similar (data not shown). Although both enzymes contain the same number of Trp residues (Espinosa V, Kettlun AM, Zanocco A, Encinas V, Cardemil E and Valenzuela MA, unpublished data), the fluorescence emission of the Pimpernel enzyme was greater than that of the Desirée apyrase at the same protein concentration.

Quenching of Trp fluorescence with acrylamide, Cs$^+$ and I$^-$

Three quenchers of different polarity were used to analyze the microenvironment of Trp residues in both apyrases. Figure 2 shows the Stern-Volmer plots for Pimpernel and Desirée enzymes both in the native and denatured form (4.8 M GndHCl) using acrylamide, a polar uncharged quencher. Both conformations showed an upward curvature for the Desirée apyrase, whereas only the denatured form presented the upward curvature for the Pimpernel enzyme. The native enzyme had a linear Stern-Volmer plot with a Ksv = 3.77.

Figure 3 provides the Stern-Volmer plots for both isoapyrases using Cs$^+$ as a quencher. A downward curvature was observed for the native Pimpernel enzyme, with a fraction of exposed residues of 0.25. On the other hand, a straight line was obtained for the denatured form with a Ksv = 0.32. In contrast, for the

Desirée enzyme linear Stern-Volmer plots were obtained for both forms of enzymes with Ksv = 0.494 and 0.976 for the native and denatured forms, respectively.

I$^-$ is a highly hydrated quencher. Figure 4 presents the Stern-Volmer plots of I$^-$ quenching. The denatured Pimpernel enzyme showed an upward curvature. The native Pimpernel apyrase exhibited a downward curvature with a fraction of exposed Trp of 0.5, whereas both forms of the Desirée enzyme exhibited linear Stern-Volmer plots with a Ksv of 3.02 and 1.15 for the denatured and the native proteins, respectively.

Discussion

The difference in maximum emission wavelength, 340 nm for Pimpernel and 336 nm for Desirée, indicates that the Trp residues are slightly more exposed in the Pimpernel apyrase. Another relevant difference between the enzymes is the higher Trp emission observed for the Pimpernel enzyme when compared with the Desirée apyrase, although both of them contain the same number of Trp residues (Espinosa V, Kettlun AM, Zanocco A, Encinas V, Cardemil E and Valenzuela MA, unpublished data). These data suggest that the tryptophan residues in the Desirée enzyme are more efficiently quenched by the solvent (water), by neighboring peptide bonds and/or by the lateral chains of amino acids such as Tyr, Cys and His (12). The decrease in fluorescence intensity of both apyrases induced by the nonhydrolyzable substrate analog (AMP-PCP) indicates that upon the nucleotide binding the local environment of
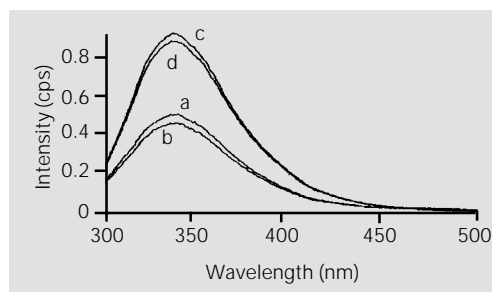

Figure 1 - Effect of ADP phosphonate on the emission spectra of Pimpernel and Desirée apyrases. The excitation wavelength was 286 nm for the four spectra. The added protein was 60 µg and 54 µg for the Desirée and Pimpernel enzymes, respectively, and 1.4 µM ADP phosphonate was added in 100 mM MES. Spectrum a: Desirée apyrase; spectrum b: Desirée apyrase + ADP phosphonate; spectrum c: Pimpernel apyrase, and spectrum d: Pimpernel apyrase + ADP phosphonate.
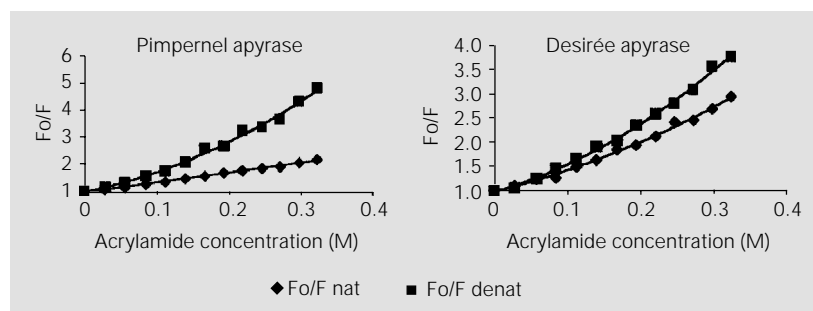
Figure 2 - Stern-Volmer plots of acrylamide quenching of Pimpernel and Desirée native (nat) and denatured (denat) apyrases. The emission intensity was measured at 340 and 336 nm for Pimpernel and Desirée, respectively. Protein concentration was 0.06 mg/ml in 100 mM MES for both enzymes. The addition of 4.8 M GndHCl produced the denatured forms. Corrections were made for dilution upon acrylamide addition. Fo: Fluorescence emission in the absence of quencher; F: fluorescence emission in the presence of quencher.
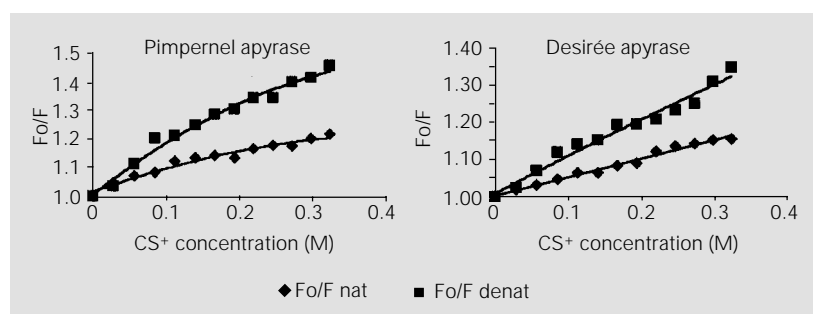

Figure 3 - Stern-Volmer plots of Cs^+ quenching of Pimpernel and Desirée native (nat) and denatured (denat) apyrases. The emission intensity was measured at 340 and 336 nm for Pimpernel and Desirée, respectively. The protein concentration was 0.06 mg/ml in 100 mM MES for both enzymes. The addition of 4.8 M GndHCl produced the denatured forms. Corrections were made for dilution upon Cs^+ addition. Fo: Fluorescence emission in the absence of quencher; F: fluorescence emission in the presence of quencher.
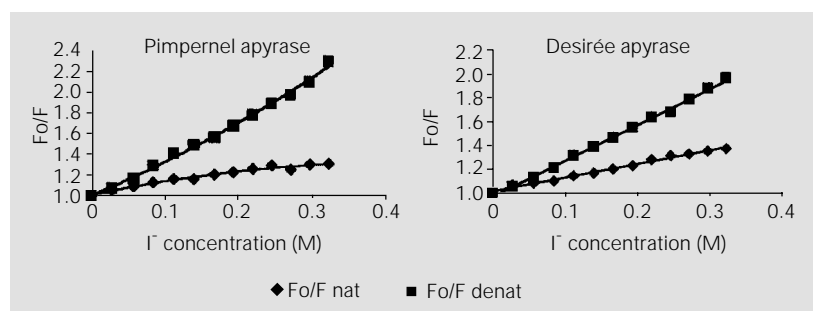

Figure 4 - Stern-Volmer plots of I^- quenching of Pimpernel and Desirée native (nat) and denatured (denat) apyrases. The emission intensity was measured at 340 and 336 nm for Pimpernel and Desirée, respectively. The protein concentration was 0.06 mg/ml in 100 mM MES for both enzymes. The addition of 4.8 M GndHCl produced the denatured forms. Corrections were made for dilution upon I^- addition. Fo: Fluorescence emission in the absence of quencher; F: fluorescence emission in the presence of quencher.

Trp varies. These results indicate conformational changes in the neighborhood of Trp in terms of nucleotide binding in both the Pimpernel and Desirée enzymes. Since acrylamide is a polar but uncharged quencher, it can probably collide with both exposed and nonexposed residues (13). The quenching experiments show the contribution of static quenching (upward curvature) for both the native and denatured forms of Desirée apyrase and for the denatured form of the Pimpernel enzyme, while the native Pimpernel apyrase has no contribution of static quenching and there is only one population of Trp equally quenched by acrylamide, thus with a linear Stern-Volmer plot.

Although the efficiency of Cs^+ in quenching indole groups is low, it may collide with the exposed or with the Trp in a negative environment. A downward curvature in the Stern-Volmer plot indicates that more than one population of fluorophores are present and that they have different accessibility to the quencher (14). On the basis of the Stern-Volmer plots, we conclude that the native Pimpernel enzyme has Trp populations with different accessibility to Cs^+, whereas denaturation of the Pimpernel enzyme induces a conformational change that renders all Trp equally accessible to Cs^+. On the other hand, both Desirée apyrase conformations have one population of Trp residues accessible to Cs^+. According to the Ksv values, Trp residues are more exposed to the quencher in the denatured form.

I^- can only interact with the exposed Trp residues. The denatured Pimpernel conformation exhibits contributions of static quenching (Stern-Volmer plot with upward curvature). The native Pimpernel apyrase has half of its Trp residues accessible to I^- and the other half does not collide with this quencher. In contrast, both forms of the Desirée enzyme show one population of Trp residues equally exposed to I^-. Since the Ksv value is higher (3.02) than the one obtained for the native form (1.15), the Trp residues

of the denatured form are more exposed to I⁻. All the quenching experiments led us to conclude that the microenvironment of the Trp residues of the two isoapyrases is different. The changes in the Stern-Volmer plot of the native forms of both Pimpernel and Desirée isoenzymes upon nucleotide binding (ADP-PCP and AMP-PCP) induce conformational changes that alter the microenvironments of the Trp residues in/near the nucleotide-binding site. The present results indicate that the nucleotide-binding site of Pimpernel apyrase is different from the nucleotide-binding site of Desirée apyrase, possibly explaining the kinetic variations.

Acknowledgments

References

1. Thomas C, Sun Y, Naus K, Lloyd A & Roux S (1999). Apyrase functions in plant phosphate nutrition and mobilizes phosphate from extracellular ATP. Plant Physiology, 119: 543-552.

2. Battastini AMO, Emanuelli T, Koester MR, Wink MR, Bonan CD, Dias RD & Sarkis JJF (1998). Studies on the anchorage of ATP diphosphohydrolase in synaptic plasma membranes from rat brain. International Journal of Biochemistry and Cell Biology, 30: 669-678.

3. Valenzuela MA, Kettlun AM, Sandoval S, García L, Mancilla M, Neckelmann G, Chayet L, Alvarez A, Cuevas F, Collados L, Espinosa V, Traverso-Cori A, Bravo I, Acevedo CG & Aranda E (1996). Comparison of the biochemical properties, regulation and function of ATP-diphosphohydrolase from human placenta and rat kidney. Brazilian Journal of Medical and Biological Research, 29: 589-597.

4. Vasconcelos EG, Ferreira ST, de Carvalho TMU, de Souza W, Kettlun AM, Mancilla M, Valenzuela MA & Verjovski-Almeida S (1996). Partial purification and immunohistochemical localization of ATP diphosphohydrolase from Schistosoma mansoni. Journal of Biological Chemistry, 271: 22139-22145.

5. Valenzuela MA, Kettlun AM, Mancilla M, Calvo V, Fanta N & Traverso-Cori A (1988). The effect of bivalent metal ions on ATPase-ADPase activities of apyrase from Solanum tuberosum. Phytochemistry, 27: 1981-1985.

6. Kettlun AM, Uribe L, Calvo V, Silva S, Rivera J, Mancilla M, Valenzuela MA & Traverso-Cori A (1982). Properties of two apyrases from Solanum tuberosum. Phytochemistry, 21: 551-558.

7. Kettlun AM, Leyton M, Valenzuela MA, Mancilla M & Traverso-Cori A (1992). Identification and subcellular localization of two isoenzymes of apyrase from Solanum tuberosum. Phytochemistry, 31: 1889-1894.

8. Mancilla M, Kettlun AM, Valenzuela MA & Traverso-Cori A (1984). Structural studies of two apyrases from Solanum tuberosum. Phytochemistry, 23: 1397-1400.

9. Kettlun AM, Uribe L, Silva S, Rivera J, Valenzuela MA & Traverso-Cori A (1981). Chemical modification of aromatic, acid and basic amino acids of two isoenzymes of apyrase from Solanum tuberosum. Archivos de Biologia y Medicina Experimentales, 14: 171-175.

10. Espinosa V, Kettlun AM, Zanocco A, Cardemil E & Valenzuela MA (1999). Fluorescence studies of ATP diphosphohydrolase from Solanum tuberosum var. Desirée. Phytochemistry (in press).

11. Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227: 680-685.

12. Chen Y & Barkley MD (1998). Toward understanding tryptophan fluorescence in proteins. Biochemistry, 37: 9976-9982.

13. Efnick MR & Ghiron CA (1981). Fluorescence quenching studies with proteins. Analytical Chemistry, 114: 199-227.

14. Lacowitcz JR (1983). Principle of Fluorescence Spectroscopy. Plenum Press, New York.