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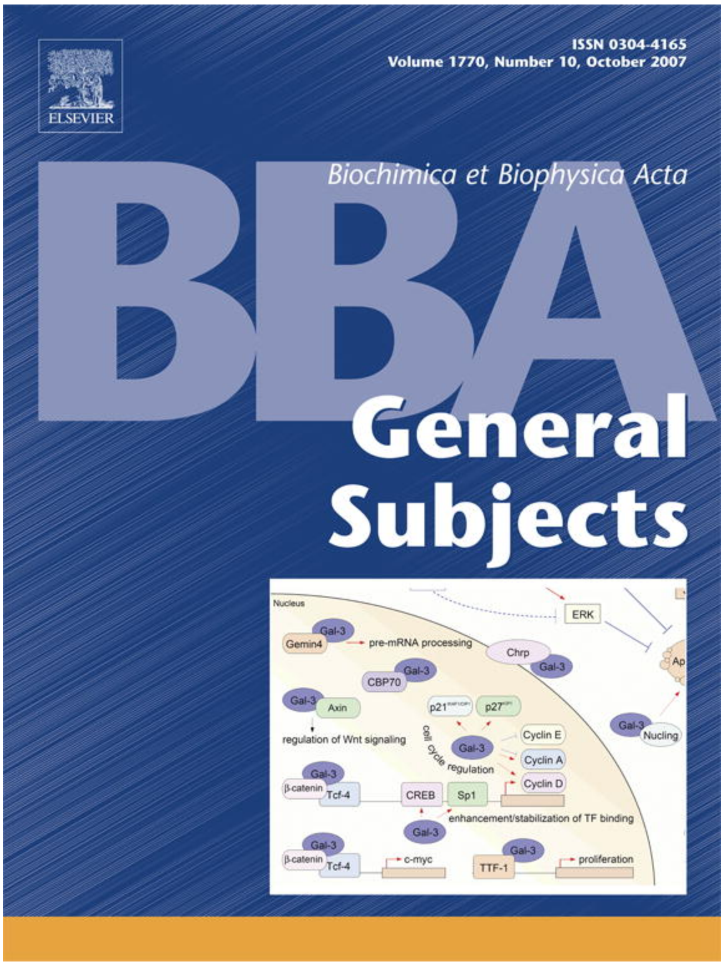

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Characterization of the adenine nucleoside specific phosphorylase of *Bacillus cereus*

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Abstract

Adenosine phosphorylase, a purine nucleoside phosphorylase endowed with high specificity for adenine nucleosides, was purified 117-fold from vegetative forms of *Bacillus cereus*. The purification procedure included ammonium sulphate fractionation, pH 4 treatment, ion exchange chromatography on DEAE-Sephacel, gel filtration on Sephacryl S-300 HR and affinity chromatography on N^6-adenosyl agarose. The enzyme shows a good stability to both temperature and pH. It appears to be a homohexamer of 164±5 kDa. Kinetic characterization confirmed the specificity of this phosphorylase for 6-aminopurine nucleosides. Adenosine was the preferred substrate for nucleoside phosphorolysis (k_{cat}/K_m 2.1×10^6 s^{-1} M^{-1}), followed by 2′-deoxyadenosine (k_{cat}/K_m 4.2×10^5 s^{-1} M^{-1}). Apparently, the low specificity of adenosine phosphorylase towards 6-oxopurine nucleosides is due to a slow catalytic rate rather than to poor substrate binding.

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Keywords: Adenosine phosphorylase; Purine nucleoside phosphorylase; Purine nucleosides; *Bacillus cereus*

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1. Introduction

Nucleoside phosphorylases are ubiquitous enzymes catalyzing the reversible phosphorolytic cleavage of the N-glycosidic bond of (2′-deoxy)ribonucleosides, to form the free nucleobase and (2′-deoxy)ribose 1-phosphate. They have been grouped into two structurally distinct protein families called nucleoside phosphorylase-I (NP-I) and nucleoside phosphorylase-II (NP-II) [1]. Members of NP-I family are either trimeric or hexameric enzymes, accepting as substrates a variety of purine nucleosides as well as the pyrimidine nucleoside uridine. NP-II family includes pyrimidine nucleoside specific enzymes showing a dimeric quaternary structure.

Purine nucleoside phosphorylase (PNP, E.C. 2.4.2.1) is undoubtedly the best known member of the NP-I family. PNP has been until now purified and characterized from a wide variety of both prokaryotic and eukaryotic species: mammalian PNPs show a homotrimeric structure, while most bacterial PNPs have generally been reported as either homotrimers or homohexamers. Some other PNPs depart from this classification, displaying either dimeric or tetrameric quaternary structures. Structural and kinetic properties of PNPs have been extensively reviewed [2].

PNPs have long been considered as enzymes involved in the salvage of purine nucleobases, because in standard conditions the equilibrium of reaction is markedly shifted towards nucleoside synthesis [3–5]. However, the biochemical role of these enzymes should rather be considered catabolic. In fact, *in vivo* nucleoside phosphorolysis is highly favoured over synthesis, since both reaction products may be further metabolized: the purine base may either be salvaged through the action of hypoxanthine-guanine phosphoribosyltransferase or oxidized to uric acid by xanthine dehydrogenase; the pentose moiety, released as either ribose 1-phosphate or deoxyribose 1-phosphate, may in turn be rapidly channelled into the intermediate metabolism, providing an additional energy source to the cell [6,7]. Moreover, bacterial PNPs are often inducible enzymes belonging to operons or regulons encoding nucleoside catabolizing enzymes [8–10].

The substrate specificity of trimeric PNPs is generally restricted to 6-oxopurine nucleosides, while hexameric enzymes

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often show a broader specificity, accepting both 6-oxo and 6-aminopurine nucleosides. However, there are some exceptions to this type of sorting because some enzymes show subunit compositions other than trimeric or hexameric [2]. Based on the present knowledge, we can only say that mammalian and yeast PNPs as well as some protozoan and bacterial enzymes are active towards 6-oxopurine nucleosides [2,11,12], while other bacterial PNPs [2] as well as PNP of the protozoan parasite *Trichomonas vaginalis* [13] show a broader specificity, since they catalyze phosphorolysis of both 6-oxo and 6-aminopurine nucleosides.

On the other hand, some organisms possess two PNPs differing in their substrate specificities. Generally, both enzymes catalyze phosphorolysis of 6-oxopurine nucleosides, while one of them can also act on adenine nucleosides. Thus, in *Escherichia coli* PNP, the product of *deoD* gene, catalyzes phosphorolysis of hypoxanthine, guanine and adenine nucleosides [14]. The second phosphorylase, encoded by the *xapA* gene, has been improperly referred to as xanthosine phosphorylase: it shows a substrate specificity similar to that of PNP, with the difference that it can also catalyze phosphorolysis of xanthosine. This enzyme does not act on adenine nucleosides [15]. Similarly, in *Bacillus stearothermophilus* one enzyme (PuNPase I) catalyzes phosphorolysis of inosine and guanosine but not of adenosine [16], while the second phosphorylase (PuNPase II) shows a broader specificity, accepting as substrates both adenine and 6-oxopurine nucleosides [17].

Interestingly, in some other organisms possessing two PNPs, including both prokaryotes and invertebrates, specificities of the two enzymes do not overlap: phosphorolysis of adenine nucleosides is catalyzed by a specific enzyme commonly referred to as adenosine phosphorylase (AdoP), while the activity of PNP is restricted to 6-oxopurine nucleosides. The presence of an AdoP activity distinguishable from PNP by pH-activity curves and inhibition studies was firstly reported in extracts of the parasitic worm *Schistosoma mansoni* [18]. However, proteins were not physically separated. Soon afterwards, phosphorolytic activity towards adenine nucleosides was detected by Senesi et al. in both vegetative forms and spores of several *Bacillus* species, the highest levels being observed in *Bacillus cereus* extracts [19]. The same authors were the first to show that in *B. subtilis* phosphorolysis of adenine nucleosides is catalyzed by a specific enzyme (AdoP) which was partially separated from PNP by ammonium sulphate fractionation and gel filtration [4]. Both phosphorylases of *B. subtilis* have been purified and characterized [20].

AdoP activity was also found in a variety of *Mycoplasma*, *Spiroplasma* and *Acholeplasma* species [21]. A 102- to 108-kDa nucleoside phosphorylase endowed with high specificity towards adenine nucleosides was purified from *Acholeplasma laidlawii* [22]. Testing AdoP activity proved to be very useful in the detection of mycoplasma contamination of animal cells and biological samples [21,23].

Finally, Trembacz and Jezewska detected AdoP activity in a number of snails and their trematode parasites [24] as well as in another trematode, the mammalian parasite *Fasciola hepatica* [25]. The same authors reported the partial purification of AdoP from hepatopancreas of the snail *Helix pomatia* and described some of its molecular and kinetic properties [26]. The enzyme was separated from PNP and appears highly specific for adenine nucleosides. It has a molecular mass of 71 kDa, a value lower than other adenosine cleaving phosphorylases. It must be noted that AdoP of *H. pomatia* is the only adenine nucleoside specific phosphorylase so far purified from eukaryotes.

In this paper we report the purification and preliminary characterization of AdoP from the vegetative forms of *B. cereus*. This study has been intended as the first step of a more detailed research aimed at identifying the structural determinants of its peculiar substrate specificity.

2. Materials and methods

2.1. Enzymes and reagents

Nucleosides, nucleobases, ribose-1-phosphate, deoxyribose-1-phosphate, adenosine deaminase (EC 3.5.4.4), xanthine oxidase (EC 1.1.3.22), DEAE-Sephacel, Sephacryl S-300 HR, N^6-5′-AMP agarose and molecular weight markers for gel filtration chromatography were from Sigma-Aldrich. The SDS-PAGE Low Range Molecular Weight Standards kit was obtained from BioRad. Recombinant bovine 5′-nucleotidase (EC 3.1.3.5) was prepared according to Allegrini et al. [27]. All other chemicals were of reagent grade.

2.2. Bacterial strain and growth conditions

B. cereus strain NCIMB 8122 (ATCC 10702) was grown in Terrific Broth (Fluka) at 30 °C under vigorous shaking from 1 ml cell suspensions stored at −20 °C in 1 M KCl containing 10% glycerol. A 100-ml culture of logarithmically growing cells was inoculated into a 4-l fermenter and grown at 30 °C under vigorous mixing with an air flow of 5.4 l/min. Late logarithmic phase cells were harvested by centrifugation at 3700×*g*, suspended in a small volume of 1 M KCl containing 10% glycerol and rapidly frozen at −20 °C to avoid sporulation.

2.3. Enzyme activities

One enzyme unit (U) catalyzes the formation of 1 μmol of product per minute at 37 °C. AdoP synthetic activity was routinely assayed by the adenosine deaminase coupled spectrophotometric method described by Miech et al. [18], following the decrease in absorbance at 265 nm associated with the conversion of adenine and ribose 1-phosphate into inosine ($\Delta\varepsilon = -6.4\ \mathrm{mM^{-1}\ cm^{-1}}$). The standard assay mixture contained 67 μM adenine and 125 μM ribose-1-phosphate in 0.1 M Tris–HCl buffer, pH 7.5. Activity of adenosine deaminase (EC 3.5.4.4) was assayed spectrophotometrically according to Koch and Vallee [28] by following the change in absorbance at 265 nm which accompanies the conversion of adenosine into inosine ($\Delta\varepsilon = -8.1\ \mathrm{mM^{-1}\ cm^{-1}}$). Phosphorolytic activity of PNP (EC 2.4.2.1) was assayed according to Jensen and Nygaard [14] in 0.1 M sodium phosphate buffer, pH 7.5, containing 2 mM inosine: the hypoxanthine formed from inosine was converted into uric acid in the presence of excess xanthine oxidase; the increase in absorbance at 293 nm was monitored ($\Delta\varepsilon = +11.3\ \mathrm{mM^{-1}\ cm^{-1}}$).

2.4. Protein content

Proteins were determined according to Bradford [29], using the Bio-Rad protein assay kit (Bio-Rad Laboratories) and bovine serum albumin as standard.

2.5. Preparation of N^6-adenosyl agarose

N^6-adenosyl agarose was prepared by enzymatic dephosphorylation of commercial N^6-5′-AMP agarose (where the C6 amino group of 5′-AMP is bound to agarose through an 11-carbon spacer arm): 5 ml of N^6-5′-AMP agarose suspension were equilibrated in 50 mM Tris–HCl buffer, pH 7.5, containing 0.5 M KCl and 40 mM $MgCl_2$ and incubated for 2 h in the presence of 1 unit of recombinant bovine 5′-nucleotidase (EC 3.1.3.5). After extensive washing with 50 mM Tris–HCl buffer, pH 7.5, the N^6-adenosyl agarose suspension was stored at 4 °C in 20% ethanol.

2.6. Ultrafiltration

Dialysis and concentration of enzyme samples were performed by ultrafiltration on an Amicon cell equipped with a PLGC membrane (molecular weight cut off 10 kDa, Sigma-Aldrich) under nitrogen atmosphere at 4 °C against an appropriate buffer.

2.7. SDS-PAGE

Electrophoresis under denaturing conditions was performed on 12% slab gels in the presence of 0.1% sodium dodecylsulphate, according to Weber and Osborn [30]. Gels were run at a constant current of 35 mA.

3. Results

3.1. Purification of adenosine phosphorylase

A summary of the purification procedure is shown in Table 1. *B. cereus* cells were suspended (40% w/v) in 50 mM Tris–HCl buffer, pH 7.5 and disrupted by ultrasonic treatment (Vibracell VCX400, Sonics and Materials). The homogenate was centrifuged 20 min at 31,000×*g* and the supernatant (crude extract of Table 1) subjected to ammonium sulphate fractionation between 51% and 72% saturation. The 72% saturation precipitate was suspended in 100 mM citrate buffer, pH 4, and incubated 15 min at 4 °C under continuous stirring. After 20 min centrifugation at 24,000×*g* the supernatant was neutralized with 1 M Tris and dialyzed against 10 mM Tris–HCl buffer, pH 7.5.

The dialyzed sample was applied on a DEAE-Sephacel column (3.2 × 6 cm) equilibrated with 10 mM Tris–HCl buffer, pH 7.5 (Fig. 1). In order to eliminate the unadsorbed material, the column was extensively washed with the same buffer at a flow rate of 12.8 ml cm^{-2} h^{-1}. Adsorbed proteins were eluted with a linear gradient of 0 to 0.45 M NaCl. Peak activity of AdoP appeared at 0.3 M NaCl. The active fractions (effluent volume of 108 to 139 ml) were pooled and concentrated by ultra-filtration.

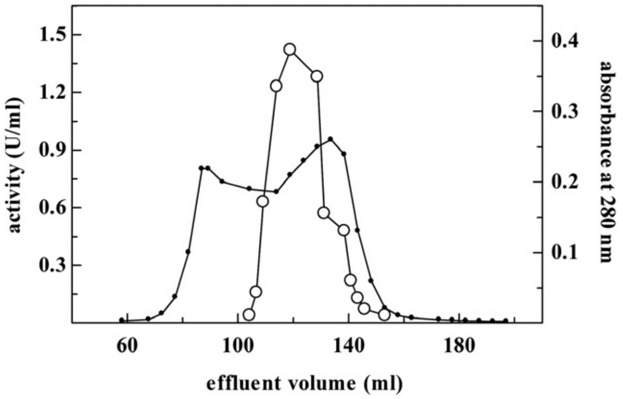

Fig. 1. Ion exchange chromatography of AdoP on DEAE-Sephacel. (●) Absorbance at 280 nm; (○) AdoP activity.

The concentrated sample was subjected to gel filtration on a Sephacryl S-300 HR column (3.2 × 83 cm) equilibrated with 50 mM Tris–HCl buffer, pH 7.5 (Fig. 2). Elution was performed with the same buffer at a flow rate of 12.8 ml cm^{-2} h^{-1}.

Finally, the active fractions of the gel filtration step (effluent volume of 365 to 414 ml) were concentrated and subjected to affinity chromatography on N^6-adenosyl agarose (Fig. 3). The concentrated sample was applied to a 1.5 × 2.3 cm column equilibrated with 50 mM Tris–HCl buffer, pH 7.5 (a phosphate free buffer was necessary to avoid the possibility for the affinity ligand to be cut off from agarose by AdoP during chromatography). After extensive washing with the same buffer containing 0.3 M NaCl at a flow rate of 35 ml cm^{-2} h^{-1} in order to eliminate the unadsorbed material, AdoP was selectively eluted with 15 mM adenosine. The active fractions (effluent volume of 5.5 to 12 ml) were pooled, dialyzed against 50 mM Tris–HCl buffer, pH 7.5, to remove NaCl and adenosine, and stored at −20 °C.

The described procedure resulted in a purification of AdoP of 117 folds (Table 1). The final preparation showed a single band after SDS-PAGE (Fig. 4) and was devoid of adenosine deaminase and PNP activities. Adenosine deaminase was in fact rapidly inactivated during preparation of the crude homogenate, since it is unstable in the absence of monovalent cations [19,31]. Concerning PNP, we can reasonably exclude the presence of appreciable amounts of contaminating PNP in purified AdoP: the marked

Table 1
Purification of adenosine phosphorylase from *Bacillus cereus*

Purification step	Protein (mg)	Activity (U [a])	Specific activity (U mg^{-1})	Purification (fold)	Yield (%)
Crude extract	464.0	31	0.07	1	100
Ammonium sulphate [b]	101.0	24	0.24	3	77
pH 4 treatment	20.0	23	1.15	16	74
DEAE Sephacel	7.3	23	3.15	45	74
Sephacryl S300 HR	4.1	19	4.63	66	61
N^6-adenosyl agarose	1.1	9	8.18	117	29

[a] One enzyme unit (U) catalyzes the formation of 1 μmol of adenosine per minute at 37 °C.

[b] Since adenosine phosphorylase is inhibited by sulphate ions, activity of the ammonium sulphate fraction was assayed after extensive dialysis against 50 mM Tris–HCl buffer, pH 7.5.

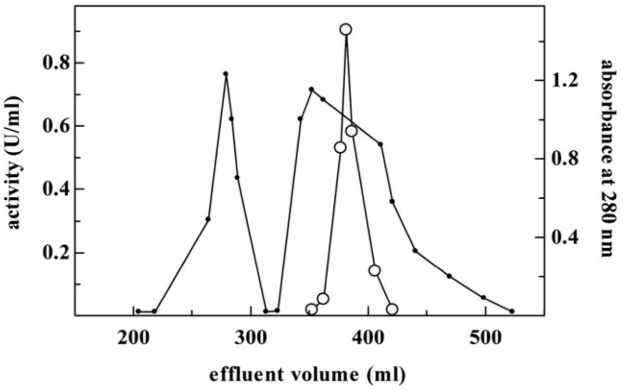

Fig. 2. Gel filtration of AdoP on Sephacryl S-300 HR. (●) Absorbance at 280 nm; (○) AdoP activity.

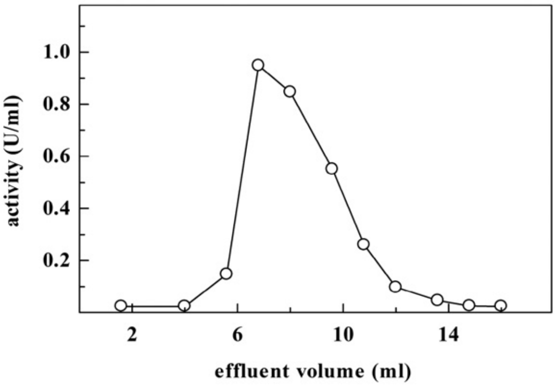

Fig. 3. Affinity chromatography of AdoP on N^6-adenosyl agarose. The chromatogram shows the elution profile of AdoP activity, starting from the addition of adenosine. The protein elution profile is not shown because of the high absorbance of adenosine present in the elution buffer. Fractions were assayed for AdoP activity after conversion of the nucleoside to inosine by adenosine deaminase.

difference in molecular masses between the two enzymes leads PNP (95 kDa) to be separated from AdoP during the gel filtration step; moreover, since PNP does not bind to N^6-adenosyl agarose (data not shown), any residual PNP activity is eliminated during the extensive washing which precedes the selective elution of AdoP. Finally, no evident protein bands corresponding to the molecular mass of PNP subunits (24 kDa) were detected after SDS-PAGE of purified AdoP (see Fig. 4).

3.2. Properties of adenosine phosphorylase

AdoP stability was studied on a partially purified enzyme preparation obtained by ammonium sulphate fractionation followed by ion exchange chromatography (see the purification procedure described above for details), in order to obtain preliminary information potentially useful for handling the enzyme as well as to further define the purification procedure.

AdoP shows a good thermal stability. The enzyme preparation was incubated for 10 min in 0.1 M Tris–HCl buffer, pH 7.5, at temperatures ranging from 20 to 70 °C and then rapidly chilled. Enzyme remained fully active up to 55 °C, while it was gradually inactivated at higher temperatures. Complete inactivation was achieved at 70 °C. Also, enzyme activity remained unchanged after 90 min at 50 °C. Thermal stability was not apparently affected by the purification procedure: purified AdoP can be stored frozen for at least 1 year without appreciable loss of activity.

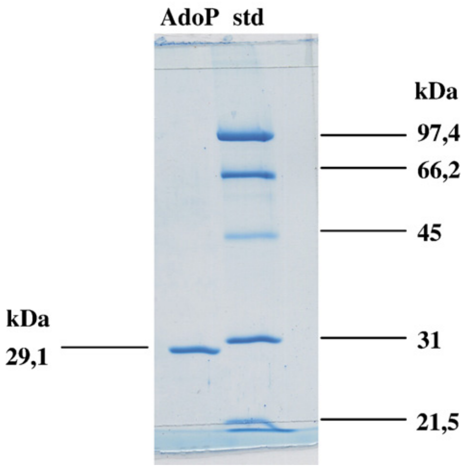

Fig. 4. SDS-PAGE of purified AdoP. The enzyme was applied on a 12% slab gel. Marker proteins (std) were: soya trypsin inhibitor, 21.5 kDa; carbonic anhydrase, 31 kDa; ovalbumin, 45 kDa; bovine serum albumin, 66.2 kDa; glycogen phosphorylase b, 97.4 kDa.

AdoP is also stable over a wide pH range: partially purified enzyme remained fully active when kept at 4 °C for 8 days at pH values ranging from 4.0 to 9.0. The enzyme still retained the original activity between pH 7 and pH 8.5 after 45 days. A pH 4 treatment was then profitably included in the purification procedure (see Table 1).

The molecular mass of native AdoP was estimated according to Andrews [32] by gel filtration on a Sephacryl S-300 HR column (1.6 × 95 cm) equilibrated with 50 mM Tris–HCl buffer, pH 7.5. Elution was performed at a flow rate of 11 ml cm^{-2} h^{-1}. AdoP activity eluted as a sharp symmetrical peak of 164 ± 5 kDa (Fig. 5). Determination of the molecular mass under denaturing conditions was carried out by SDS-PAGE according to Weber and Osborn [30]. The enzyme shows a single band of 29.1 kDa after Coomassie blue staining (Fig. 4), consistent with a homohexameric structure of native AdoP.

Table 2 shows the substrate specificity of AdoP. Phosphorolysis of nucleosides was measured at the indicated wavelengths by following the absorbance change associated with the conversion of each nucleoside (adenosine, 2′-deoxyadenosine, guanosine, 2′-deoxyguanosine, cytidine, 2′-deoxycytidine, uridine, and thymidine) into the corresponding nucleobase; activities towards inosine, 2′-deoxyinosine and xanthosine were measured according to the xanthine oxidase coupled assay (see Materials and methods). AdoP exhibits maximal activity with adenosine, followed by 2′-deoxyadenosine, while inosine and 2′-deoxyinosine are very poor substrates. Reactions were strictly dependent on the presence of phosphate: no change in absorbance could be detected with both adenine and hypoxanthine nucleosides when phosphate was omitted from the reaction mixture, ruling out the presence of any hydrolytic activity in the enzyme preparation. No activity could be detected when xanthosine, guanine nucleosides or pyrimidine nucleosides were tested as substrates.

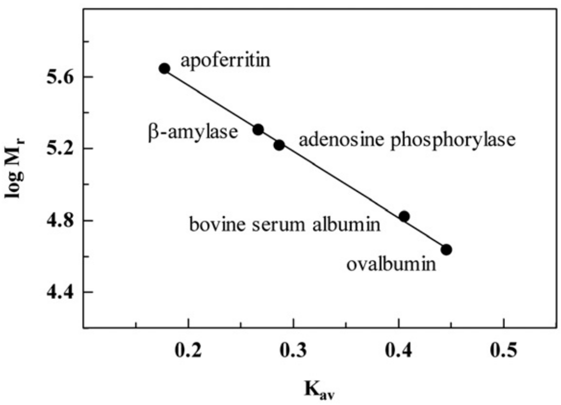

Fig. 5. Determination of the molecular mass of native AdoP. The following molecular weight marker proteins were used: ovalbumin, 43 kDa; bovine serum albumin, 66 kDa; β-amylase, 200 kDa; apoferritin, 443 kDa.

Table 2
Substrate specificity of adenosine phosphorylase

Nucleoside	Wavelength (nm)	Δε (mM^{-1} cm^{-1})	Relative activity (%)
Adenosine	260	−1.2	100
2′-Deoxyadenosine	260	−1.6	46.7
Inosine	293	+11.3	1.5
2′-Deoxyinosine	293	+11.3	1.7
Guanosine	248	−3.8	nd
2′-Deoxyguanosine	260	−4.3	nd
Xanthosine	293	+7.4	nd
Cytidine	260	−5.0	nd
2′-Deoxycytidine	260	−4.8	nd
Uridine	260	−1.8	nd
Thymidine	260	−1.4	nd

Reactions were carried out in quadruplicate in 80 mM Tris–HCl buffer, pH 7.5, containing 8.5 mM Na_2HPO_4 and 0.15 mM nucleoside, and started by adding purified AdoP (25 ng/ml). Phosphorolysis of nucleosides was measured at the indicated wavelengths by following the absorbance change associated with the conversion of each nucleoside to the corresponding nucleobase. Activities towards inosine, 2′-deoxyinosine and xanthosine were measured according to the xanthine oxidase coupled assay (see Materials and methods). Changes in extinction coefficients at the indicated wavelengths were determined at 25 °C in Tris–HCl buffer, pH 7.5. For each nucleoside maximal rates of phosphorolysis were reported as a percentage of activity towards adenosine. nd = activity not detected.

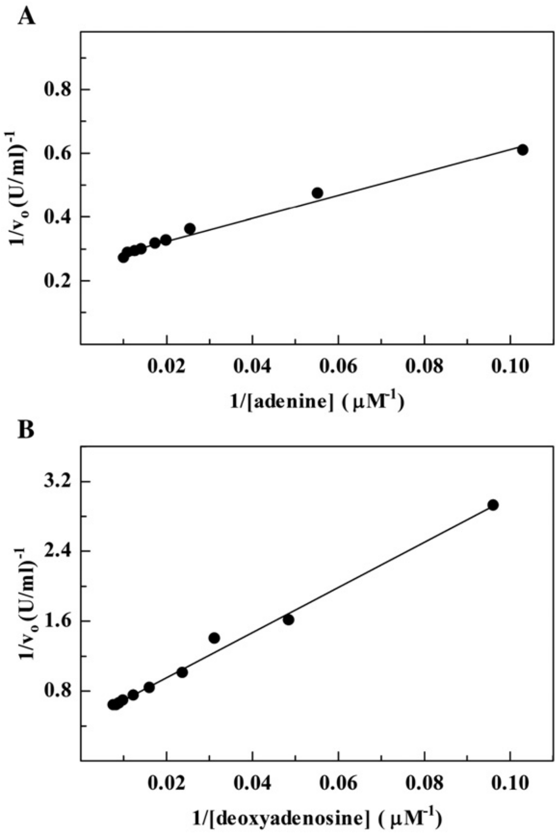

Fig. 6. Panel A, Lineweaver–Burk plot of AdoP activity for nucleoside synthesis. Enzyme activity was measured with adenine as variable substrate at fixed 0.13 mM ribose-1-phosphate concentration according to the adenosine deaminase coupled assay described under Materials and methods. Panel B, Lineweaver–Burk plot of AdoP activity for deoxyadenosine phosphorolysis. Activity was measured in the presence of 100 mM sodium phosphate buffer, pH 7.5, by following the decrease in absorbance at 260 nm ($\Delta\varepsilon = -1.2\ mM^{-1}\ cm^{-1}$).

AdoP shows Michaelis–Menten kinetics: double reciprocal plots of initial velocity vs. substrate concentration appear to be linear for both phosphorolysis and synthesis reactions. As an example, Fig. 6 shows Lineweaver–Burk plots of AdoP activity with adenine (Panel A) and deoxyadenosine (Panel B) as variable substrates. Similar results were obtained with adenosine, hypoxanthine and deoxyribose-1-P.

Kinetic parameters of AdoP for both phosphorolytic and synthetic reactions are reported in Table 3. Values for adenine and hypoxanthine nucleosides were obtained from double reciprocal plots: phosphorolysis reactions were carried out in quadruplicate in the presence of 100 mM sodium phosphate buffer, pH 7.5. Nucleoside concentrations were in the ranges 10 to 130 μM, 20 to 300 μM and 20 μM to 1 mM for adenine nucleosides, inosine and 2′-deoxyinosine, respectively. We selected concentration ranges for hypoxanthine nucleosides wider than for adenine nucleosides in view of the low activity of AdoP towards hypoxanthine nucleosides, so as to minimize errors in collecting the data and hence in determining K_m and k_{cat}. Phosphorolysis of adenine nucleosides was measured by following the decrease in absorbance at 260 nm ($\Delta\varepsilon = -1.2$ and $-1.6\ mM^{-1}\ cm^{-1}$ for adenosine and 2′-deoxyadenosine, respectively); phosphorolysis of hypoxanthine nucleosides was measured according to the xanthine oxidase coupled assay described in Materials and methods.

Kinetic parameters for adenine, ribose 1-P and deoxyribose 1-P were determined according to Price and Stevens [33] from secondary plots where values of $1/V_{max}$ (obtained from primary plots of initial velocity vs. concentration of a given substrate at various fixed concentrations of the second substrate) were reported as a function of the reciprocal of concentration of the second substrate. V_{max} and K_m for the fixed substrate were obtained from the intercepts on the $1/V_{max}$ axis and the fixed substrate axis, respectively. Nucleoside synthesis was assayed according to the adenosine deaminase coupled method (see Materials and methods). Kinetic parameters for adenine were obtained from 10 primary plots of initial velocity vs. ribose 1-P concentration (5 to 65 μM) carried out at fixed adenine concentrations ranging between 12 and 98 μM; kinetic parameters for ribose 1-phosphate were obtained from 8 primary plots with adenine as the variable substrate (2 to 100 μM) and ribose 1-phosphate as the fixed substrate (22 to 127 μM); finally, kinetic parameters for 2-

Table 3
Kinetic parameters of adenosine phosphorylase

	k_{cat} [a] (s^{-1})	K_m (M)	k_{cat}/K_m (s^{-1} M^{-1})
Nucleoside phosphorolysis			
Adenosine	55.4	27×10^{-6}	2.1×10^6
2′-Deoxyadenosine	25.9	62×10^{-6}	4.2×10^5
Inosine	0.8	55×10^{-6}	1.5×10^4
2′-Deoxyinosine	0.9	110×10^{-6}	8.3×10^3
Nucleoside synthesis			
Adenine	33.8	32×10^{-6}	1.1×10^6
Ribose 1-P	38.0	29×10^{-6}	1.3×10^6
2-Deoxyribose 1-P	232.1	380×10^{-6}	6.1×10^5

[a] k_{cat} values were calculated by assuming six active sites per enzyme molecule and a molecular mass of 29.1 kDa per subunit.

deoxyribose 1-phosphate were obtained from 6 primary plots with adenine as the variable substrate (3.5 to 88 μM) and ribose 1-phosphate as the fixed substrate (49 to 308 μM).

4. Discussion

We purified to electrophoretic homogeneity AdoP from vegetative cells of *B. cereus* and studied some of its kinetic and molecular properties. The enzyme is stable over a wide range of pH values and shows a good thermal stability. Molecular mass determination under native and denaturing conditions indicates that AdoP is a 164±5 kDa protein formed by six identical subunits of 29.1 kDa; so, it may be assigned to the hexameric phosphorylase group of the NP-1 family [1]. Specificity of purified AdoP was tested by assaying its phosphorolytic activity on a number of purine and pyrimidine nucleosides. The enzyme appears to be specific for adenine nucleosides. Hypoxanthine nucleosides were phosphorolysed at a very low rate, while no activity could be detected with other purine and pyrimidine nucleosides. Kinetic parameters were determined for both phosphorolytic and synthetic reactions; k_{cat}/K_m ratios confirm the preference of AdoP for 6-aminopurine nucleosides and also suggest that the low specificity of the enzyme towards inosine and deoxyinosine is due to a low catalytic capacity (low turnover numbers) rather than to a poor efficiency in nucleoside binding (K_ms for hypoxanthine and adenine nucleosides are in the same order of magnitude). Both k_{cat} and K_m for 2-deoxyribose 1-P appear to be higher than the corresponding parameters obtained for ribose 1-P. In this regard, we note that the k_{cat}/K_m ratio for 2-deoxyribose 1-P is in line with that for 2′-deoxyadenosine, indicating a preference of AdoP for ribose containing substrates with respect to deoxyribose containing substrates.

Comparing our present data on *B. cereus* AdoP with properties of PNP purified from the same micro-organism allow us to point out that the two phosphorylases markedly differ in both subunit composition and substrate specificity. Indeed, PNP is a 95-kDa protein formed by four identical subunits of 24 kDa [34] which catalyzes phosphorolysis of hypoxanthine and guanine nucleosides but not of adenine nucleosides [8]. The two phosphorylases of *B. cereus* may be compared with the corresponding enzymes purified from *B. subtilis*. In this regard, AdoP and PNP of *B. subtilis* appear to be very similar to the corresponding enzymes of *B. cereus* in both substrate specificity and molecular arrangement [20]. However, it must be noted that AdoP of *B. cereus* obeys Michaelis–Menten kinetics (see Fig. 6), while the kinetic behaviour of *B. subtilis* AdoP shows a downward curvature of Lineweaver–Burk plot with both phosphate and deoxyadenosine as variable substrates [20]. Complex, non-linear kinetics have often been documented in both mammalian and bacterial PNPs. Such behaviours have been referred to as substrate activation or negative cooperativity. However, an adequate model allowing a clear interpretation of experimental data has not yet been presented [2].

Among other phosphorylases active on adenine nucleosides, AdoP of *B. cereus* may be compared with PuNPase II of *B. stearothermophilus* and PNP of *E. coli*. These enzymes catalyze phosphorolysis of both 6-oxo and 6-aminopurine nucleosides with different specificities: adenosine appears to be the primary substrate of *B. stearothermophilus* PuNPase II followed by 2′-deoxyadenosine [17], while PNP of *E. coli* is highly active towards deoxyinosine, even though it can efficiently catalyze phosphorolysis of adenine nucleosides as well [14]. Therefore, in terms of substrate specificity, PuNPase II seems to be closer to AdoP than PNP of *E. coli*. On the contrary, being a high molecular mass hexamer, AdoP appears to be more similar, in terms of molecular arrangement, to the 138-kDa hexameric PNP of *E. coli* [14] than to PuNPase II of *B. stearothermophilus*, a 107-kDa tetramer [17].

Very recently, the structure of one of the two *Bacillus anthracis* gene products annotated as PNPs in the genomic sequence databases has been published [35]. The protein was referred to as the product of the *DeoD* gene and appears as a disc-shaped hexamer resembling *E. coli* PNP (nearly 60% sequence identity). However, it cannot be identified with any of the two putative PNPs of *B. anthracis* because it has not been subjected to kinetic characterization. Since *B. anthracis* is strictly related to *B. cereus*, we can only speculate that this protein might show a good similarity with AdoP.

The physiological role of AdoP has not yet been fully clarified. Generally, PNPs have either a broad specificity, acting on both adenine and 6-oxopurine nucleosides (PNP of *E. coli*), or they do not cleave at all 6-aminopurine nucleosides (PNPs of mammalian cells). The presence of a phosphorylase specific for adenine nucleosides in organisms so heterogeneous such as mycoplasmas, bacilli and snails is indeed not easy to explain. This may be related to the roles played in different organisms by phosphorylases endowed with different specificities. Thus, in snails it has been postulated that AdoP may be involved in catabolism of nucleic acids derived from alimentary substances during the active period of life, also contributing to maintain high ATP levels during the winter sleep [26].

In bacteria, nucleoside phosphorylases seem to play a key role in nucleoside catabolism. In fact, many micro-organisms are able to grow on nucleosides as a carbon source. Utilization of these compounds generally involves deamination by adenosine deaminase or cytidine deaminase, cleavage of N-glycosidic bond catalyzed by a nucleoside phosphorylase and isomerization of the pentose 1-phosphate to the corresponding pentose 5-phosphate catalyzed by phosphopentomutase. Ribose 5-phosphate enters the pentose phosphate pathway, while deoxyribose 5-phosphate is specifically cleaved by deoxyriboaldolase into glyceraldehyde 3-phosphate and acetaldehyde. So, purine nucleosides can be utilized as an energy source by channelling their pentose moieties into the intermediate metabolism [6,9,36].

In enterobacteria, enzymes and transport proteins involved in nucleoside utilization are all induced in the presence of nucleosides in the growth medium. Their expression is regulated in a complex manner, including both negative and positive control mechanisms [9]. In this way, exogenous adenine nucleosides are utilized through the action of PNP either directly or following deamination catalyzed by adenosine deaminase.

This metabolic pattern is somewhat different in bacilli, some of which do not possess adenosine deaminase. Thus, in *B. subtilis* genes encoding nucleoside catabolizing enzymes are grouped in two operons, one including phosphopentomutase and PNP, the other including deoxyriboaldolase, pyrimidine phosphorylase and a carrier of pyrimidine nucleosides [37]. Both operons are negatively regulated by repressor proteins and are sensitive to catabolite repression [38–41]. In *B. subtilis*, AdoP is the only enzyme acting upon adenine nucleosides, because this micro-organism lacks adenosine deaminase [19]. However, AdoP is encoded by a single gene and its expression is unresponsive to the presence of nucleosides in the external medium [37]. This would not allow a rapid utilization of exogenous adenine nucleosides. On the contrary, other purine nucleosides can be rapidly utilized through the action of the inducible PNP. Unfortunately, the role of AdoP in *B. subtilis* has not been further investigated.

A peculiar role of AdoP in the mechanism regulating expression of nucleoside catabolizing enzymes has been postulated in *B. cereus* [6]. In this micro-organism utilization of purine nucleosides involves adenosine deaminase and PNP, whose action makes the pentose moiety of nucleosides available to be further catabolized through the phosphopentomutase and, in the case of deoxyribose moiety, deoxyriboaldolase reactions. These enzymes are all induced in the presence of nucleosides in the growth medium. They are also subjected to catabolite repression when glucose is added to the growth medium. Moreover, adenosine deaminase is induced by adenine and adenine nucleosides, while it is repressed by inosine [10,42,43]. On the contrary, AdoP is constitutively expressed in *B. cereus*. Its activity cannot be detected in intact cells, unless adenosine deaminase is repressed [10]. Thus, when catabolic enzymes are induced, adenine nucleosides are mainly utilized through deamination followed by phosphorolysis catalyzed by PNP, rather than through direct phosphorolysis catalyzed by AdoP. In this context, the role of AdoP would be to signal, through the formation of adenine, the presence in the medium of nucleosides to be utilized as energy source: adenine will cause induction of adenosine deaminase, leading to the formation of inosine which in turn triggers induction of the other enzymes involved in the pathway.

One might speculate that AdoP of *B. cereus* could have evolved from a PNP endowed with a broad specificity, which lost the ability to phosphorolyse 6-oxopurine nucleosides, in the meantime acquiring a novel function in regulating the expression of nucleoside catabolizing enzymes. In this context, it will be attractive to study the structure of AdoP in detail. Comparing amino acid sequence and molecular arrangement of AdoP with other nucleoside phosphorylases could contribute to understand the structural determinants of its substrate specificity, also helping to better understand its role in nucleoside catabolism.

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