Structural basis of the substrate specificity of *Bacillus cereus* adenosine phosphorylase

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Purine nucleoside phosphorylases catalyze the phosphorolytic cleavage of the glycosidic bond of purine (2′-deoxy)nucleosides, generating the corresponding free base and (2′-deoxy)ribose 1-phosphate. Two classes of PNPs have been identified: homotrimers specific for 6-oxopurines and homohexamers that accept both 6-oxopurines and 6-aminopurines. *Bacillus cereus* adenosine phosphorylase (AdoP) is a hexameric PNP; however, it is highly specific for 6-aminopurines. To investigate the structural basis for the unique substrate specificity of AdoP, the active-site mutant D204N was prepared and kinetically characterized and the structures of the wild-type protein and the D204N mutant complexed with adenosine and sulfate or with inosine and sulfate were determined at high resolution (1.2–1.4 Å). AdoP interacts directly with the preferred substrate through a hydrogen-bond donation from the catalytically important residue Asp204 to N7 of the purine base. Comparison with *Escherichia coli* PNP revealed a more optimal orientation of Asp204 towards N7 of adenosine and a more closed active site. When inosine is bound, two water molecules are interposed between Asp204 and the N7 and O6 atoms of the nucleoside, thus allowing the enzyme to find alternative but less efficient ways to stabilize the transition state. The mutation of Asp204 to asparagine led to a significant decrease in catalytic efficiency for adenosine without affecting the efficiency of inosine cleavage.

1. Introduction

Purine nucleoside phosphorylases (PNPs; EC 2.4.2.1) catalyze the reversible phosphorolysis of purine (2′-deoxy)nucleosides, generating the corresponding purine base and (2′-deoxy)ribose 1-phosphate. PNPs play a key role in nucleotide metabolism, in which phosphorolysis of the N-glycosidic bond allows the purine nucleobase to be salvaged for nucleotide biosynthesis via phosphoribosyltransferase reactions, thus allowing energy savings with respect to de novo purine biosynthesis. Ribose 1-phosphate can either be utilized in the de novo or salvage pathways, via conversion to 5-phosphoribosyl 1-pyrophosphate, or can be used as a source of energy through the formation of intermediates that can enter the glycolysis and the citric acid cycle either directly or via the pentose phosphate pathway (Bzowska et al., 2000; Tozzi et al., 2006).

PNPs are members of the NP-I family of nucleoside phosphorylases, which share a common α/β-subunit fold (Pugmire & Ealick, 2002). On the basis of substrate specificity, molecular mass, subunit composition and amino-acid sequence, PNPs can be further divided into two subfamilies consisting of...
homotrimeric or homohexameric. Trimeric PNPs are generally specific for 6-oxopurines and have been found in eukaryotes and some prokaryotes. Hexameric PNPs are characterized by broad substrate specificity, accepting both 6-oxopurine and 6-aminopurine nucleosides, and are mainly found in prokaryotes, but have also been found in some eukaryotic parasites. The hexameric PNPs from *Thermus thermophilus* (Tahirow et al., 2004) and *Plasmodium falciparum* (Daddona et al., 1986) are exceptions because they do not accept adenosine as a substrate.

*Bacillus cereus* expresses two PNPs: one is specific for 6-oxopurines (Gardner & Kornberg, 1967) and the other is specific for 6-aminopurines (Sgarrella et al., 2007). The latter enzyme is the focus of this work and is referred to as adenosine phosphorylase (AdoP). *B. cereus* AdoP is a constitutive enzyme (Tozzi et al., 1981). Previous work has reported that, together with the constitutive enzyme 5'-nucleotidase, it is involved in the utilization of the sugar moiety of exogenous AMP, adenosine and inosine as an energy source. Formation of adenosine causes the induction of adenosine deaminase, thus acting as a signal to the cells that adenine nucleosides are available as an energy source (Tozzi et al., 1981). A specific adenosine phosphorylase activity, distinct from the inosine-guanosine phosphorylase activity, has also been detected in other members of the genus *Bacillus* (Senesi et al., 1977).

AdoP has been purified and characterized from *B. subtilis* (Jensen, 1978; Senesi et al., 1976). AdoP from *B. cereus* is 100% identical to AdoP from *B. anthracis*, the causative agent of anthrax and a well known bioterrorism agent (Atlas, 1998). *B. anthracis* is genetically and phenotypically related to *B. cereus* (Ivanova et al., 2003; Read et al., 2003). Other organisms in which a distinct adenosine phosphorylase has been identified are *Schistosoma mansoni* (Miech et al., 1975), *Acholeplasma laidlawii* (McElwain et al., 1988), *Helix pomatia* (Trebacz & Jezewska, 1993) and *Fasciola hepatica* (Trebacz & Jezewska, 1998).

While *B. cereus* AdoP accepts both 6-aminopurine and 6-oxopurine nucleoside substrates, kinetic studies revealed a 100-fold preference ($k_{cat}/K_m$) for adenosine over inosine (Sgarrella et al., 2007). The enzyme has a molecular weight of 164 kDa for the hexamer and 29.1 kDa for the protomer, of 164 kDa for the hexamer and 29.1 kDa for the protomer, with high sequence identity (>45%) compared with other hexameric PNPs. Structures of *B. anthracis* AdoP (Grenha et al., 2005) and *B. cereus* AdoP (PDB entry 2ac7; A. Rinaldo-Matthis, S. Allegrini & F. Sgarrella, unpublished work) have previously been reported.

To investigate the structural basis of the peculiar substrate specificity of *B. cereus* AdoP, we constructed, purified and characterized the active-site mutant D204N. While the efficiency for adenosine was reduced, that for inosine increased slightly, effectively reversing the substrate specificity. We determined several crystal structures, including those of wild-type *B. cereus* AdoP, its complexes with either adenosine and sulfate or inosine and sulfate, and the D204N mutant complexed with either adenosine and sulfate or inosine and sulfate. The crystals diffracted to high resolution ranging from 1.2 to 1.4 Å. Correlation between structural data and kinetic measurements provides insights into the unique substrate preferences of *B. cereus* AdoP.

## 2. Experimental procedures

### 2.1. Site-directed mutagenesis

Site-directed mutagenesis was performed on the *B. cereus* AdoP gene cloned in pET5b vector (Novagen; F. Sgarrella, private communication), referred to in the following as pET5b/AdoP. A modification of the two-stage PCR method described by Ekici et al. (1997) was employed to prepare the D204N mutant. A sense mutagenic primer was designed on the sequence of the AdoP gene, starting at base 601 and containing the triplet encoding the desired mutation (in bold): 5'-ACAGTAAGTATCATCTTCACT-3'. The sense and antisense specific primers 5'-TGCGAGAATCATGAGTTGCATATTGAAAGC-3' and 5'-CTTGAATTCATATTGTTGAGATTGCTGCATC-3' were the same as used for the cloning of the AdoP gene and included *NdeI* and *EcoRI* restriction sites, respectively (in bold). In the first polymerase chain reaction (PCR), a 25 µl mixture consisting of 30 pmol sense mutagenic primer, 15 pmol antisense specific primer, 15 ng pET5b/AdoP DNA template, 200 µM of each dNTP, 1 mM MgSO$_4$ and 1.25 U Platinum *Pfx* DNA polymerase (Invitrogen) in 1× PCR buffer was incubated at 367 K for 2 min; 30 consecutive cycles were then performed, each consisting of denaturation at 367 K for 30 s, annealing at 325 K for 30 s and extension at 341 K for 30 s, followed by a final incubation at 341 K for 90 s. The megaprimer obtained in this reaction was purified from agarose gel and used as an antisense primer in a second PCR reaction together with 60 pmol sense specific primer, 15 ng pET5b/AdoP, 200 µM of each dNTP, 1 mM MgSO$_4$ and 1.25 U Platinum *Pfx* DNA polymerase in 50 µl 1× PCR buffer. The PCR conditions were the same as those used in the first PCR reaction, with 1 min of extension in each cycle. The purified mutant gene was cloned in pCR1-TOPO (Invitrogen) and then digested with *NdeI* and *EcoRI* endonucleases, and finally ligated in pET5b vector that had previously been digested with the same restriction enzymes. The resulting recombinant plasmid, designated pET5b/D204N, was sequenced on both strands at Genelab ENEA Casaccia, S. M. di Galeria (Rome, Italy) to confirm the presence of the mutation.

### 2.2. Expression and purification

The wild-type and mutant proteins were overexpressed in *Escherichia coli* BL21 (DE3) and purified to homogeneity using a modification of the protocol described by Sgarrella et al. (2007). The cells were grown at 310 K and induced with 0.4 mM isopropyl β-D-1-thiogalactopyranoside for 3 h after which they reached an OD$_{600}$ of 0.6. The cells were harvested by centrifugation at 3000 g for 8 min at 277 K, after which the cell pellet was resuspended in 50 mM Tris–HCl pH 7.5 and lysed using a Vibra-Cell VC-400 Ultrasonic Processor (Sonic & Materials). Cell debris was removed by centrifugation at 31 000 g for 45 min at 277 K. The crude extract was subjected to ammonium sulfate fractionation (between 46.2 and 77%...
## Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

<table>
<thead>
<tr>
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<td><strong>Beamline</strong></td>
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<td>23-BM</td>
<td>24-ID-C</td>
<td>24-ID-C</td>
<td>8-BM</td>
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<td>0.9997</td>
<td>0.97946</td>
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<td>0.97949</td>
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<td>P₆₃22</td>
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<td>93.5 (77.8)</td>
<td>99.2 (100)</td>
<td>99.7 (100)</td>
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<td>13.1 (5.4)</td>
<td>3.4 (2.0)</td>
<td>7.1 (7.1)</td>
<td>8.2 (8.0)</td>
</tr>
<tr>
<td><strong>No. of unique reflections</strong></td>
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<td>88363 (7949)</td>
<td>88185 (7217)</td>
<td>58861 (5806)</td>
<td>59410 (5858)</td>
</tr>
<tr>
<td><strong>Total No. of reflections</strong></td>
<td>301266</td>
<td>1160800</td>
<td>295688</td>
<td>415665</td>
<td>485298</td>
</tr>
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</table>

saturation). The precipitate obtained was resuspended in 50 mM Tris–HCl pH 7.5 and further purified using a Sephacryl S300 HR column (Amersham Biosciences; 1.6 × 95 cm) pre-equilibrated with 50 mM Tris–HCl pH 7.5. The fractions containing the enzymatic activity were pooled together and loaded onto an N⁶-adenosyl agarose affinity column (1.5 × 2.3 cm). N⁶-Adenosyl agarose was prepared by dephosphorylation of 5'-AMP-agarose (Sigma) with bovine cytosolic 5'-nucleotidase II (Allegrini et al., 1997). The column was washed with 0.3 M NaCl in 50 mM Tris–HCl pH 7.5. The wild-type enzyme was eluted with 10 mM adenosine and 0.3 M NaCl in 50 mM Tris–HCl pH 7.5. For the D204N mutant, 10 mM inosine replaced the adenosine in the elution buffer. The purified proteins were dialyzed against 50 mM Tris–HCl pH 7.5 using a 10 kDa molecular-weight cutoff cellulose membrane (Sigma).

During purification, protein concentration was measured by the Bradford assay (Bradford, 1976), while the purified AdoP concentration was determined by measuring the absorbance at 205 nm (Scopes, 1974; ε(1 mg ml⁻¹) = 29.161, calculated for the D204N mutant). The enzymatic activity of recombinant AdoP was detected spectrophotometrically. The synthesis of adenosine from 88 μM adenine and 90 μM ribose 1-phosphate in 50 mM Tris–HCl pH 7.5 was monitored at 265 nm (Δε = 6.39 M⁻¹ cm⁻¹) in a coupled assay using 0.6 μM isoenzyme deaminase (Miech et al., 1972). Inosine phosphorolytic activity was measured at 293 nm (Δε = 11.26 M⁻¹ cm⁻¹) in a coupled assay using 1 μM inosine, 100 mM NaH₂PO₄/Na₂HPO₄ buffer pH 7.5 and 0.1 U ml⁻¹ xanthine oxidase (Kaleckar, 1947). All enzymatic assays were performed at 310 K.

### 2.3. Determination of kinetic parameters

Phosphorolytic activity towards adenosine and inosine was measured as described by Sgarrella et al. (2007). Continuous spectrophotometrical assays were performed at 310 K using an excess of phosphate (100 mM NaH₂PO₄/Na₂HPO₄ buffer pH 7.5) in 500 μl reaction mixtures. Adenosine phosphorolytic activity was measured directly at 256.5 nm (Δε = 1.812 M⁻¹ cm⁻¹) at 175 μM using 3.13 mM ml⁻¹ D204N mutant or 3.74 mM ml⁻¹ wild-type AdoP. Inosine phosphorolytic activity was measured at 293 nm (Δε = 11.26 M⁻¹ cm⁻¹) with a coupled assay using 0.1 U ml⁻¹ xanthine oxidase, 2.88 mM ml⁻¹ D204N mutant or 7.76 mM ml⁻¹ wild-type AdoP and 12 different inosine concentrations varying from 13 μM to 1 mM (Sgarrella et al., 2007). The kinetic parameters were analyzed by non-linear regression data fitted to the Michaelis–Menten equation.

### 2.4. Crystallization

Proteins were concentrated to ~9 mg ml⁻¹ in 10 mM Tris–HCl pH 7.5 by ultrafiltration. Crystallization experiments were performed at 291 K using the hanging-drop vapor-diffusion method, with the drop consisting of 1 μl protein solution plus 1.5 μl reservoir solution and equilibrated against 400 μl reservoir solution. The optimal crystallization conditions were 1.3–1.5 M ammonium sulfate for the wild-type enzyme and 1.1–1.25 M sodium citrate pH 5.2–5.5 for the D204N mutant. Despite the different crystallization conditions, the wild type and the D204N mutant shared the same crystal morphology. Doubly terminated hexagonal crystals grew in about 4 d to typical dimensions of 150 × 150 × 200 μm. These crystals belonged to space group P₆₃22 (average unit-cell parameters a = 122, c = 68 Å), with one protomer per asymmetric unit and a Matthews coefficient (Matthews, 1968) of 2.8 Å³ Da⁻¹, which corresponds to a solvent content of 57%. Complexes of the wild-type protein and the D204N mutant with ligands were obtained by soaking experiments. The stabilization solutions were similar to the mother liquor except 7–15% more concentrated. Owing to crystal fragility, gradual soaking was often necessary and the ligands adenosine (Ado) or inosine (Ino) were increased in five steps to final concentrations of 1 and 10 mM, respectively. For D204N complexes, 7–14 m M ammonium sulfate was also added to the soaking solution. Soaking for 1 h was sufficient for the ligands to bind.

Prior to freezing, the crystals were cryoprotected by the addition of 18–20% (v/v) glycerol to the mother liquor or the
soaking solution. Crystals were frozen by quickly plunging them into liquid nitrogen.

2.5. Data collection and processing

Data sets were collected on beamlines 23-BM (MAR 225 detector), 8-BM (Quantum 315 detector) and 24-ID-C (Quantum 315 detector) at the Advanced Photon Source (APS). A total of 30–60° of data were collected from each crystal with an oscillation range of 0.5°. Data were processed using HKL-2000 (Otwinowski & Minor, 1997). Data-processing statistics are shown in Table 1.

2.6. Structure determination and refinement

Wild-type and D204N mutant AdoP structures were determined by molecular replacement using the CNS software package (Brünger et al., 1998). A protomer of the E. coli PNP structure (PDB entry 1ecp; Mao et al., 1997) was used as the search model in the absence of a better homology structure at the time. The initial rigid-body refinement and simulated annealing resulted in a model with an R factor of 37%. Based on a composite OMIT map, the protein backbone required minor changes and the side chains were manually adjusted using the graphics programs O (Jones et al., 1991) and Coot (Emsley et al., 2010). The structures were refined by alternating cycles of simulated-annealing, energy-minimization and B-factor refinement using CNS and manual rebuilding. All of the complexes showed clear Fo−Fc difference density for the ligands, which were directly built into each of the structures. Solvent molecules were added after ligand building and refined by energy minimization as well as temperature-factor refinement. The water molecules were validated by checking the distance and geometry of the hydrogen bonds. In each structure, 10–13% of the residues were built in alternate conformations. The N-terminal methionine is missing in all of the structures, while residues 207–217 and 232–234 exhibited varying degrees of disorder. In the AdoP–SO₄ and AdoP–Ino–SO₄ complexes, residues 208–213 were not built in the model, while in the other structures this region was built with temperature factors above average values. The final rounds of refinement were performed using PHENIX (Adams et al., 2011). All structures were refined to Rw and Rfree values of below 15% and 17%, respectively, at which point the AdoP–Ado–SO₄ and AdoP–Ino–SO₄ complexes, both at 1.2 Å resolution, were further subjected to anisotropic refinement and converged with final Rw and Rfree values of below 12.5%/14.1% and of 12.8%/14.8%, respectively. More than 90% of the residues were in the most favored regions of the Ramachandran plot for all of the structures and no residues were in the disallowed region. Refinement statistics are shown in Table 2. Figures were created using MolScript (Kraulis, 1991), Raster3D (Merritt & Bacon, 1997) and PyMOL (DeLano, 2002).

3. Results

3.1. Kinetics of the D204N mutant

The D204N mutant was prepared to assess the contribution of Asp204 to catalysis and binding of the substrates in B. cereus AdoP. Asp204 is conserved in hexameric PNPs, but in trimeric PNPs it is replaced by asparagine (Asn243 in human PNP), which is essential for the phosphorolysis of 6-oxopurines (Erión, Stoeckler et al., 1997; Erion, Takabayashi et al., 1997). Table 3 shows the kinetic parameters determined for wild-type B. cereus AdoP and the D204N mutant with adenosine and inosine as substrates. Mutating Asp204 to asparagine resulted in a 2.2-fold increase in catalytic efficiency towards inosine, with a 1000-fold change in substrate preference; while the wild-type enzyme prefers adenosine over inosine 100-fold, the D204N mutant exhibits a ninefold preference for inosine over adenosine.

3.2. Overall structure of B. cereus AdoP

The overall structure of B. cereus AdoP is similar to those of E. coli PNP (Mao et al., 1997) and other hexameric PNPs (Appleby et al., 2001; Grenha et al., 2005; Schnick et al., 2005;
Table 3

Kinetic parameters of wild-type and D204N mutant AdoP with adenosine and inosine as substrates.

<table>
<thead>
<tr>
<th></th>
<th>$K_m$ (μM)</th>
<th>$k_{cat}$ (s⁻¹)</th>
<th>$k_{cat}/K_m$ (s⁻¹ M⁻¹)</th>
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</thead>
<tbody>
<tr>
<td>Wild type</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Adenosine</td>
<td>23 ± 6</td>
<td>75 ± 5</td>
<td>3.1 × 10⁴</td>
</tr>
<tr>
<td>Inosine</td>
<td>315 ± 52</td>
<td>9.3 ± 0.5</td>
<td>3.0 × 10⁶</td>
</tr>
<tr>
<td>D204N</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenosine</td>
<td>24 ± 7</td>
<td>0.18 ± 0.01</td>
<td>7.3 × 10³</td>
</tr>
<tr>
<td>Inosine</td>
<td>102 ± 11</td>
<td>6.8 ± 0.2</td>
<td>6.6 × 10⁴</td>
</tr>
</tbody>
</table>

Shi et al., 2004; Tahir et al., 2004; Zang et al., 2005). It should be noted that the space group of *B. cereus* AdoP in our studies was determined to be P6₃22 with one protomer per asymmetric unit, while the deposited structure (PDB entry 2ac7) was reported to have the same unit-cell parameters but in space group P6₃ with two protomers per asymmetric unit. A check of the deposited data using phenix.xtriage (Zwart et al., 2005) showed a strong twofold axis with an $R_{merge}$ of 3.3%, suggesting that the correct space group for PDB entry 2ac7 is also P6₃22. The *B. cereus* AdoP hexamer is disc-shaped, with dimensions of approximately 100 × 100 × 36 Å (Fig. 1). The hexamer has 32 symmetry and can be thought of as a trimer of dimers (AB, CD and EF). Each dimer contains a pair of twofold-related active sites. The dimers are joined by B–C, D–E and F–A interfaces, which are far away from the active sites. Extensive contacts at the B–C, D–E and F–A interfaces involve $\alpha_3$, $\alpha_6$, $\beta_3$, $\beta_1$ and the loop between $\alpha_3$ and $\alpha_4$. Contacts at the A–B, C–D and E–F interfaces involve helix $\alpha_2$ and loops $\beta_2$–$\beta_3$ and $\beta_3$–$\alpha_5$. In most hemaxic PNP the interface containing the active site (A–B type) buries a larger surface area (Schnick et al., 2005; Tahir et al., 2004); however, in *B. cereus* AdoP the dimer (A–B type) buries ~2800 Å² of surface area, while the second interface (B–C type) buries ~3500 Å² of surface area. The *B. cereus* AdoP protomer has the characteristic NP-I fold (Pugmire & Ealick, 2002) consisting of a single $\alpha/\beta$-domain with a central nine-stranded $\beta$-sheet forming a distorted $\beta$-barrel that is surrounded by seven $\alpha$-helices (Fig. 2).

3.3. AdoP active site

The active site was characterized using four complexes: (i) wild-type AdoP with adenosine and sulfate (AdoP–Ado–SO₄), (ii) wild type with inosine and sulfate (AdoP–Ino–SO₄), (iii) D204N with adenosine and sulfate (D204N–Ado–SO₄) and (iv) D204N with inosine and sulfate (D204N–Ino–SO₄). The active site is located at the C-terminal end of the $\beta$-barrel and can be divided into three regions: the phosphate-binding site, the ribose-binding site and the base-binding site (Fig. 3). The phosphate-binding and ribose-binding sites are identical in all of our AdoP structures, while important differences occur in the base-binding site depending on the substrate.

The phosphate-binding site is occupied by a sulfate ion in all of the complexes, as widely used in the determination of both trimeric and hexameric PNP since it shares similar chemical properties with the phosphate ion and is an inhibitor of the phosphorolysis reaction (Appleby et al., 2001; Mao et al., 1997; Tahir et al., 2004). Gly20, Arg87, Thr90 and Arg43* (where the asterisk indicates a residue from the adjacent subunit) make hydrogen bonds to the sulfate O atoms. The sulfate ion also donates hydrogen bonds to the ribose O3' and O2'. In the binary complex, two water molecules replace the ribose O atoms.

In the ribose-binding site, the 5'-hydroxyl group of the sugar hydrogen-bonds to His4* and a water molecule. Met180 is involved in a hydrophobic interaction with the hydrophobic

Figure 1
Structure of the *B. cereus* AdoP hexamer in ribbon representation. Protomers B, C, D, E and F were generated by crystallographic symmetry from protomer A. Protomers that form two active sites at the interface are shown in similar colors (AB, CD and EF). Adenosine and sulfate are shown in red in stick representation.

Figure 2
Structure of the *B. cereus* AdoP protomer. The protomer of AdoP is shown in ribbon representation and colored by secondary structure. Adenosine and sulfate are shown in red stick representation.
face of the pentose ring and forms a hydrogen bond to the 2'-hydroxyl group of the ribose. Glu181 makes hydrogen bonds to both hydroxyl groups of the ribose. Several hydrophobic residues surround the purine ring in the base-binding site, with Val178 and Ile206 on one side and Phe159 and Met180 on the other (Fig. 3). Different interactions between the purine base and Asp204 or Asn204 were detected in the four ternary complexes in which both nucleoside and sulfate are bound in the active site. These different interactions resulted from changes in the position and orientation of Asp204 or Asn204, while the orientation in the active site of the nucleoside was same in all four complexes (Fig. 4). For the AdoP–Ado–SO₄ complex (Fig. 5a), Asp204 interacts directly with the base, making two hydrogen bonds to the N7 and N6 atoms of the adenine base. Asp204 also accepts one hydrogen bond from Ser203 and one from a water molecule. A series of water molecules form a hydrogen-bonding network with the N1 and N6 of the base and the carbonyl group of Phe159. In the AdoP–Ino–SO₄ complex (Fig. 5b) the Asp204 side chain is positioned further from the base, allowing two well ordered water molecules to bridge between Asp204 and the base. The position of Asn204 in the D204N–Ado–SO₄ complex (Fig. 5c) is very similar to the position of Asp204 in the AdoP–Ado–SO₄ complex, with two hydrogen bonds formed between Asn204 and the purine base. In the D204N–Ino–SO₄ complex (Fig. 5d), the carboxamido group of Asn204 forms a weak hydrogen bond (∼3.47 Å) to N7 of the purine base and interacts with O6 of the purine base indirectly through a water molecule, as seen in the AdoP–Ino–SO₄ complex. Another feature in common between the two complexes is the network of water molecules that connects N1 and O6 of the purine base and Phe159.

4. Discussion

4.1. Structural basis of substrate specificity

The kinetic parameters reported in Table 3 demonstrate the high specificity of B. cereus AdoP for adenosine. A comparison of the interactions between Asp204 and the purine base in the AdoP–Ado–SO₄ and AdoP–Ino–SO₄ ternary complexes may explain the structural basis of its substrate preference. When adenosine is bound in the active site (Fig. 5a), the Asp204 side chain is ∼2.76 Å away from the N7 atom of adenosine and is properly oriented to act as a proton donor and offset the partial negative charge that is transferred to the N7 position of the base from the weakening glycosidic bond (Fig. 6). Protonation of the nucleobase in the transition state is essential for leaving-group activation by a mechanism that is analogous to that of human purine nucleoside phosphorylase (Erion, Stoeckler et al., 1997; Kline & Schramm, 1993; Schramm, 1999).

In the active site of the AdoP–Ino–SO₄ complex (Fig. 5b), Asp204 interacts indirectly with the base owing to the insertion of two water molecules. In this case, a water molecule could serve as the proton donor to N7 of the purine base, but would be less efficient compared with an acidic amino-acid side chain. Alternatively, AdoP could utilize a mechanism similar to that described for the trimeric PNP from Cellulomonas for stabilizing the transition state, involving the delocalization of the electron density on the six-membered ring.
ring of inosine (Tebbe et al., 1999). In the ternary complex of *Cellulomonas* PNP with 8-iodoguanine and phosphate, the negative charge is localized at O6 of the purine base, thus forming an enolate-ion intermediate. The enolic tautomer could be stabilized by a Glu residue (Glu204), which accepts a hydrogen bond from N1 of the purine base. This Glu residue, which is structurally and functionally conserved in human PNP (Glu201) and 5'-deoxy-5'-methylthioadenosine phosphorylase (MTAP) from *Sulfolobus solfataricus* (Glu163), is located outside the active site in *B. cereus* AdoP (Glu162). Mutagenesis studies confirmed that Glu162 does not interact with the substrate (unpublished results). In the structure of the AdoP–Ino–SO₄ complex, an ordered water molecule was found to make a hydrogen bond to N1 and could help to delocalize the electron density in a circuit that involves N1, three water molecules and O6 (Fig. 5b). The strength of an N1–water hydrogen bond, however, is less than that of the strong hydrogen bond formed between N1 and Glu201 in human PNP, Glu204 in *Cellulomonas* PNP or Glu163 in *S. solfataricus* MTAP. Compared with the direct interaction of Asp204 and N7, utilizing these two alternative but less efficient ways of stabilizing the transition state could be the molecular basis for the 107-fold preference for adenosine compared with inosine.

### 4.2. Change in substrate preference of *B. cereus* AdoP

Extensive studies conducted on human PNP have already revealed the importance of the conserved asparagine residue in trimeric PNP for 6-oxopurine substrate specificity. The N243A mutant led to a 1000-fold decrease in $k_{cat}$ for inosine (Erion, Takabayashi et al., 1997). The cleavage of adenosine by wild-type human PNP is

![Figure 5](image-url)

**Figure 5**
Stereo diagrams of the base-binding site. (a) AdoP–Ado–SO₄ complex. (b) AdoP–Ino–SO₄ complex. (c) D204N–Ado–SO₄ complex. (d) D204N–Ino–SO₄ complex. C atoms are shown in gray for the protein, in light blue for adenosine and in green for inosine. N atoms are shown in blue, O atoms in red and S atoms in yellow. Water molecules are shown as red spheres and labeled HOH. Hydrogen bonds are indicated by dashed lines and labeled with distances in Å.
essentially undetectable, while 6-aminopurine nucleosides were very good substrates for the N243D mutant and were preferred over inosine, thus reversing the substrate specificity (Stoeckler et al., 1997). In addition, PNP from T. thermophilus, despite its hexameric quaternary structure normally associated with broad substrate specificity, has an asparagine residue near N7 and its selectivity for inosine is similar to that of the trimeric PNPs (Tahirov et al., 2004).

Two different mechanisms have been proposed for trimeric PNPs on the basis of key catalytic residues. Erion, Stoeckler et al. (1997) suggested a role for Asn243 in stabilizing the transition state by donating a hydrogen bond to N, while Tebbe et al. (1999) proposed that Asn243 (Asn246 in Cellulomonas PNP) supports substrate binding rather than catalysis and that Glu201 (Glu204 in Cellulomonas PNP) interacts as the key catalytic residue, as discussed in §4.1.

In our studies, mutation of Asp204 to asparagine in B. cereus AdoP resulted in a 430-fold decrease in the catalytic efficiency of adenosine cleavage. More significantly, the mutation resulted in no change in the efficiency of inosine cleavage, effectively reversing the substrate specificity. However, N204D only slightly prefers inosine, while wild-type B. cereus AdoP strongly prefers adenosine. This result is consistent with the role of asparaginase in conferring selectivity towards 6-oxo-purines and is complementary to the reversal of substrate specificity obtained with the N243D mutant of human PNP by Stoeckler et al. (1997). The structure of the D204N-Ino-SO₄ complex (Fig. 5d) provides some insights. In this complex, the distance between the carboxamide group of Asn204 and N7 of the purine base (~3.47 Å) is not favorable for hydrogen-bond formation. The negative charge on the purine base formed during the transition state could be alternatively delocalized on the six-membered ring of the base and on the network of water molecules that connect N1 and O6 of the purine base, as hypothesized for the AdoP-Ino-SO₄ complex. Consistent with this assumption is the nearly identical kcat of the wild-type enzyme and the D204N mutant towards inosine.

4.3. Comparison between E. coli PNP and B. cereus AdoP active sites

Among the structurally characterized PNPs, B. cereus AdoP, which is 100% identical to B. anthracis AdoP, shares the highest sequence identity (56%) with E. coli PNP. AdoP strongly prefers adenosine, while E. coli PNP has a similar catalytic efficiency for both adenosine and inosine (Bennett et al., 2003). Furthermore, AdoP cleaves adenosine 44-fold more efficiently than E. coli PNP. Despite their catalytic differences, the two enzymes exhibit significant similarities in overall structure as well as active-site composition and substrate-binding geometry. Superposition of B. cereus AdoP and E. coli PNP using DALI (Holm & Sander, 1998) showed a root-mean-square deviation of 1.4 Å for 234 aligned residues. The superposition also revealed that 11 of the 12 active-site residues are identical. The exception is Thr90 in the phosphate-binding site of B. cereus AdoP, which is replaced by a functionally equivalent Ser90 in E. coli PNP.

A superimposition of the active sites of the two enzymes each complexed with adenosine and sulfate is shown in Fig. 7. The major difference between the two binding sites is the conformation of the loop region from residues 207 to 217. Compared with E. coli PNP, loop 207–217 in B. cereus AdoP adopts a more closed conformation. This different positioning of loop 207–217 appears to compensate for a disruption of the N-terminal end of helix α5 in B. cereus AdoP caused by a different orientation of the Arg217 side chain with respect to the equivalent residue in E. coli PNP. As a
result, in *B. cereus* AdoP the backbone carbonyl of Glu216 is prevented from making a hydrogen bond to the backbone amide of Thr220 and contributing to the first turn of helix α5, as in the case of *E. coli* PNP. Instead, Glu216 is involved in a hydrogen bond to the side chain of Arg101 from the loop connecting βα and βα (Fig. 7). In all *B. cereus* AdoP complex structures, loop 207–217 is either partially disordered or has relatively high B factors, suggesting that this loop could be flexible during substrate binding and product release. The same region also showed different conformations from protomer to protomer in *E. coli* PNP complexes; there are three crystallographically independent protomers in the structure (Bennett et al., 2003).

Comparison of *B. cereus* AdoP with *E. coli* PNP suggests that it would be difficult to predict *a priori* the relative substrate specificities of these two types of nucleoside phosphorylases. One possibility is that the high efficiency of *B. cereus* AdoP for adenosine compared with *E. coli* PNP could be the result of a less open active site and better positioning and orientation of the catalytically important aspartic acid residue with respect to adenosine. An Asp204 to asparagine mutant has recently been reported for *E. coli* PNP; however, no structural data were reported for the mutants or their complexes (Mikleušević et al., 2011). Relative activities at fixed substrate concentrations showed significant reductions of activity towards adenosine, inosine and guanosine of approximately 800-fold, 70-fold and 30-fold, respectively. Detailed structural and kinetic studies are needed to further assess the role of residue 204 in the determination of substrate specificity for *E. coli* PNP.

5. Conclusion

The biochemical and kinetic studies reported here show that Asp204 plays an important role in the preference of *B. cereus* AdoP for adenosine. Mutation of Asp204 to asparagine results in a significant decrease in the efficiency of adenosine cleavage and a slight increase in the efficiency of inosine cleavage, thus resulting in a reversal of substrate preference. The structural data correlate with the kinetic results and suggest that AdoP and its mutants can stabilize the transition state in various ways but that these are not equally favored energetically. The most favorable geometry occurs when adenosine is bound in the active site, with Asp204 directly protonating the adenine at N7.

This work was supported by funding from the University of Sassari, Italy (FS) and by National Institutes of Health grant GM073220 (SEE). This work is based upon research conducted at the Advanced Photon Source on the Northeastern Collaborative Access Team beamlines, which are supported by award RR-15301 from the National Center for Research Resources at the National Institutes of Health. Use of the Advanced Photon Source is supported by the US Department of Energy, Office of Basic Energy Sciences under Contract No. DE-AC02-06CH11357.

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