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Adenosine binding to low-molecular-weight purine nucleoside phosphorylase: the structural basis for recognition based on its complex with the enzyme from *Schistosoma mansoni*

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Schistosomes are unable to synthesize purines *de novo* and depend exclusively on the salvage pathway for their purine requirements. It has been suggested that blockage of this pathway could lead to parasite death. The enzyme purine nucleoside phosphorylase (PNP) is one of its key components and molecules designed to inhibit the low-molecular-weight (LMW) PNPs, which include both the human and schistosome enzymes, are typically analogues of the natural substrates inosine and guanosine. Here, it is shown that adenosine both binds to *Schistosoma mansoni* PNP and behaves as a weak micromolar inhibitor of inosine phosphorolysis. Furthermore, the first crystal structures of complexes of an LMW PNP with adenosine and adenine are reported, together with those with inosine and hypoxanthine. These are used to propose a structural explanation for the selective binding of adenosine to some LMW PNPs but not to others. The results indicate that transition-state analogues based on adenosine or other 6-amino nucleosides should not be discounted as potential starting points for alternative inhibitors.

1. Introduction

Schistosoma mansoni is one of the parasitic species responsible for the disease schistosomiasis. In 2005, the World Health Organization estimated that approximately 200 million people were infected with this particular species (World Health Organization, 2005). It is known that the parasite is unable to synthesize purine bases *de novo* and therefore the purine-salvage pathway is used exclusively to supply purine bases for energy requirements as well as for DNA and RNA synthesis (Senft *et al.*, 1972; Senft & Crabtree, 1983). For these reasons, it is an attractive target for the development of selective inhibitors against schistosomiasis. Indeed, several purine analogues have been used in the past in experimental treatment of the disease (Dovey *et al.*, 1985; el Kouni *et al.*, 1987; el Kouni, 1991).

In the case of *S. mansoni*, purine nucleoside phosphorylase (SmPNP) is the only enzyme of this pathway of known three-dimensional structure (Pereira *et al.*, 2005). PNP is responsible for the phosphorolytic conversion of inosine and guanosine to ribose-1-phosphate and their respective bases. Two classes of PNP have been described. Low-molecular-weight (LMW) PNPs are trimeric; they are largely, but not exclusively, found in mammals and show substrate selectivity for inosine and guanosine but not for adenosine. The second class, the high-molecular-weight (HMW) PNPs, are principally found in bacteria and are able to phosphorylize adenosine as well as inosine and guanosine (Pugmire & Ealick, 2002). Although some reports describing phosphorolytic activity of LMW PNPs towards adenosine are available (Stoeckler *et al.*, 1997;

Table 1
Statistics of data collection and structure refinement.

	SmPNP–adenosine	SmPNP–adenine	SmPNP–inosine	SmPNP–hypoxanthine
Data-collection statistics				
Space group	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$
Unit-cell parameters (Å)	a = 49.715, b = 121.27, c = 133.24	a = 48.99, b = 119.99, c = 130.98	a = 48.82, b = 118.52, c = 129.82	a = 47.94, b = 118.20, c = 129.83
Detector	MAR Mosaic 225	MAR Mosaic 225	MAR CCD 165	MAR CCD 165
X-ray source	LNLS MX2	LNLS MX2	LNLS D03B-MX1	LNLS D03B-MX1
Wavelength (Å)	1.43	1.2	1.432	1.432
Resolution range (Å)	46.9–2.30 (2.42–2.30)	45.88–1.85	118.67–1.9 (2.0–1.9)	130.18–1.85 (1.95–1.85)
Redundancy	13.2 (13.2)	4.1 (3.9)	4.7 (4.5)	3.4 (3.3)
R_{meas} (%)	13.3 (57.2)	9.0 (41.9)	6.3 (55.1)	6.1 (38.9)
R_{merge} (%)	12.8 (57.2)	7.8 (36.5)	5.6 (48.6)	5.2 (32.8)
Completeness (%)	100.0 (100.0)	99.7 (99.3)	99.9 (99.9)	99.1 (97.8)
Total reflections	491940	275206 (37811)	730799 (39346)	216548 (29740)
Unique reflections	37280	66.688 (9588)	60248 (8662)	63249 (8928)
$I/\sigma(I)$	17.0 (4.3)	13.2 (3.1)	23.0 (2.1)	13.8 (3.3)
Refinement parameters				
R (%)	17.5	16.3	18.4	18.5
R_{free} (%)	20.4	20.3	24.2	23.4
Ramachandran plot				
Most favoured region (%)	92.0	91.3	90.9	92.2
No. of residues in disallowed regions	3	4	5	3
Overall B factor (Å^2)	36.0	29.9	38.63	26.8
Ligand B factor (Å^2)	29.92	31.92	44.51	38.37
No. of protein atoms	6237	6389	6429	6362
No. of water molecules	333	763	560	651
No. of ligand atoms	84	50	67	57
R.m.s.d. bond lengths (Å)	0.015	0.016	0.022	0.022
R.m.s.d. bond angles (Å)	1.547	1.730	1.245	1.984

Maynes *et al.*, 1999), their preference for inosine is of the order of 10^5-fold. Their lack of catalytic activity towards adenosine has recently been attributed to their inability to stabilize the negative charge on O(6) during catalysis owing to the absence of a hydrogen on N(1). This proposal was based on the crystal structure of *Cellulomonas* sp. PNP, which is able to bind adenosine but unable to catalyze phosphorolysis (Tebbe *et al.*, 1999). In summary, despite the large body of information that has accumulated over the years concerning the mechanism of action of PNPs, several details still remain to be fully clarified. These include the elucidation of the structural determinants of adenosine binding by LMW PNPs and of why some such enzymes, such as that from *Cellulomonas*, are competent in this respect and others, such as human and bovine PNPs, are not.

Since the original observation that lack of PNP activity is associated with T-cell immune deficiency (Giblett *et al.*, 1975) many studies of the potential applications of PNP inhibitors as therapeutic agents have been undertaken, including their use in the selective suppression of cellular immunity, in the treatment of malignant lymphoproliferative disorders and to counter autoimmunity (Glazer, 1984). Recently, several transition-state inhibitors of PNPs, with proposed applications in the treatment of parasitic diseases such as malaria, have been developed with K_i values in the nanomolar to picomolar range (Evans *et al.*, 2003; Schramm, 2002; Fedorov *et al.*, 2001; Clinch *et al.*, 2009). These include adenosine analogues in the case of the high-molecular-weight enzymes (Rinaldo-Matthis *et al.*, 2007). Here, we demonstrate that the schistosome enzyme, like *Cellulomonas* PNP, is capable of binding adenosine but not of catalyzing its cleavage. In addition, we describe the first crystal structures of binary complexes of an LMW PNP with both adenosine and adenine. Furthermore, in order to facilitate direct comparison with the physiological substrates, we have also determined the structures of SmPNP complexed with inosine and hypoxanthine.

2. Materials and methods

2.1. Preparation of SmPNP, crystallization and adenosine soaking

The purification and crystallization protocols for SmPNP were performed as described previously (Pereira *et al.*, 2003, 2005). All complexes were obtained by soaking. In the case of inosine, the crystals were soaked in a solution containing 20% PEG 1500, 15 m*M* sodium acetate buffer pH 4.9 or 5.0 (the pH of crystal growth), 20% glycerol and 5 m*M* inosine (initially dissolved in water). For the remaining ligands, 10% DMSO was added to the above solution and the ligands (previously dissolved in DMSO) were used at concentrations of 2.5 m*M* in the case of adenosine and of 5 m*M* for hypoxanthine and adenine. Typical soaking times were between 48 and 96 h prior to crystal freezing.

2.2. Data collection and processing

Crystals from the soaking experiments were frozen directly in liquid nitrogen in cryoloops and diffraction data were collected using synchrotron radiation at the Brazilian National Laboratory of Synchrotron Light (LNLS), Campinas, Brazil. Data from the adenosine and adenine complexes were collected on beamline W01B-MX2 using a MAR Mosaic 225

CCD detector (MAR Research), while those from the inosine and hypoxanthine complexes were collected on beamline D03B-MX1 using a MAR CCD 165 detector. In all cases data were collected in 1° increments in φ to the resolutions given in Table 1. The data were indexed and integrated using the program *MOSFLM* (Leslie, 1999) and scaled using *SCALA* from the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994).

2.3. Structure determination and refinement

All structures were initially solved using molecular replacement with either *Phaser* (McCoy, 2007) in the case of the adenosine and adenine complexes or *MOLREP* (Vagin & Teplyakov, 2000) for the remainder. The structure of SmPNP in complex with acetate (PDB code 1td1; Pereira *et al.*, 2005) was used as the search model in all cases. Crystallographic refinement was carried out using either *PHENIX* (Adams *et al.*, 2002) or a combination of *PHENIX* and *REFMAC* (Murshudov *et al.*, 1997) in the case of the hypoxanthine complex. Noncrystallographic symmetry (NCS) restraints were used in the case of the lower resolution adenosine complex and model building was performed with *Coot* (Emsley & Cowtan, 2004) using σ_A-weighted $2F_o - F_c$ and $F_o - F_c$ electron-density maps. The ligand molecules were added using the Find Ligand routine of *Coot*. Water molecules and additional fortuitous ligands such as sulfate ions and DMSO molecules were placed where appropriate using *Coot*. The behaviour of R and R_{free} was used as the principal criterion for validating the refinement protocol and its convergence. The stereochemical quality of the model was evaluated with *PROCHECK* (Laskowski *et al.*, 1993). The coordinates and structure factors have been deposited in the PDB under the following codes: 3f8w (SmPNP–adenosine), 3faz (SmPNP–inosine), 3fnq (SmPNP–hypoxanthine) and 3e9r (SmPNP–adenine).

2.4. Adenosine IC_{50} determination

The IC_{50} of adenosine for SmPNP was determined employing an automated method using a BIOMEK3000 workstation based on the modified method of Kalckar described by Erion *et al.* (1997). The assay mixture (200 µl) contained 1 m*M* iodonitrotetrazolium (INT), 20 mU xanthine oxidase, 10 µ*M* inosine, 100 m*M* Tris–HCl buffer pH 7.0 and 50 m*M* potassium phosphate pH 7.0 together with different concentrations of adenosine (400.0, 266.4, 177.4, 118.2, 78.7, 52.4 and 34.9 m*M*). The reaction was started by the addition of SmPNP (∼500 ng) and was monitored in a microplate reader at 500 nm for 2 min. All measurements were made in quadruplicate. A nonlinear four-parameter fit was used to calculate the IC_{50} value according to the sigmoidal I_{max} model using the *Sigma Plot* software.

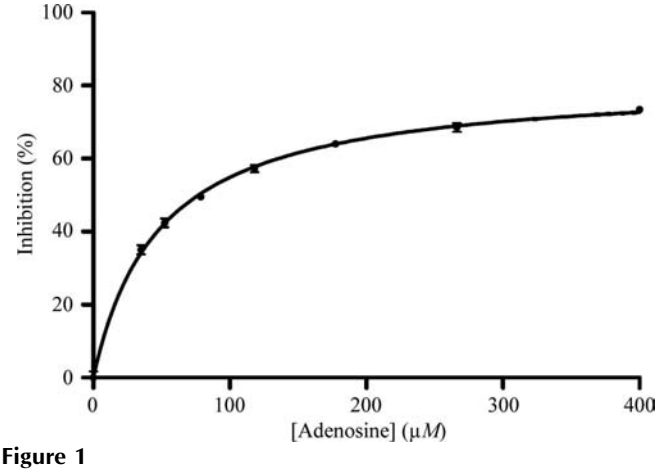

Figure 1
Inhibition of inosine phosphorolysis by adenosine.

3. Results and discussion

Like other LMW PNPs, the enzyme from *S. mansoni* was unable to catalyze the phosphorolysis of adenosine even at elevated substrate concentrations (300 µ*M*; data not shown). On the other hand, adenosine was able to bind to the enzyme and act as a weak inhibitor of inosine phosphorolysis, for which it presented an IC_{50} of 82.3 ± 0.7 µ*M* (Fig. 1). This value is comparable with the K_i reported for PNP from *Cellulomonas* sp., for which adenosine is also known to act as a weak inhibitor, a feature that has been described as distinguishing it from other PNPs (Tebbe *et al.*, 1999). It is therefore of interest to understand the basis of such inhibition and the structural differences which give rise to the ability of some LMW PNPs to bind adenosine whilst others do not.

A trimer is observed in the asymmetric unit of all the structures reported here and data-collection and refinement statistics are summarized in Table 1. Of particular note is the fact that the adenosine and adenine complexes described here are the first of their kind for LMW PNPs. The overall structures are all broadly similar to that reported previously for SmPNP bound to acetate. For example, the r.m.s.d. over all C^α atoms of the trimer on comparing the inosine and acetate complexes is 0.52 Å and that of the adenosine and inosine complexes is 0.66 Å. The only noticeable difference is the ordering of the gate region in the *B* subunit (residues 250–266) of the adenosine and inosine complexes; this region showed no interpretable electron density in the original complex with acetate (Pereira *et al.*, 2005). In the adenosine and inosine complexes this region presents readily interpretable electron density in all three subunits; it is unlikely that the difference in the behaviour of the *B* subunit has any physiological relevance.

Surprisingly, adenosine binds to SmPNP in an almost identical fashion to that observed for the natural substrate inosine. It was readily identified in all three active sites of the trimer (Fig. 2), where it forms ten hydrogen bonds, two of them with well ordered water molecules (Fig. 3). Two direct hydrogen bonds are made with Asn245, in which $O^{\delta1}$ acts as the acceptor of a hydrogen bond from the exocyclic 6-amino group and $N^{\delta2}$ donates a hydrogen bond to N(7). This probably involves flipping of the Asn245 side chain with respect to its orientation in the inosine complex (Figs. 2*b* and 4), where it is expected that Asn245 $N^{\delta2}$ donates a hydrogen bond to the

6-oxo group of the ligand (Tebbe *et al.*, 1999). In this case no second hydrogen bond can be formed between N(7) and $O^{\delta1}$ as neither is protonated. However, there is some degree of ambiguity in the current literature with respect to the orientation of the Asn245 side chain on binding 6-oxo nucleosides such as inosine since $N^{\delta2}$ could alternatively form a hydrogen bond to N(7) of the imidazole ring (Bzowska *et al.*, 2000). However, the scheme proposed above involving a hydrogen

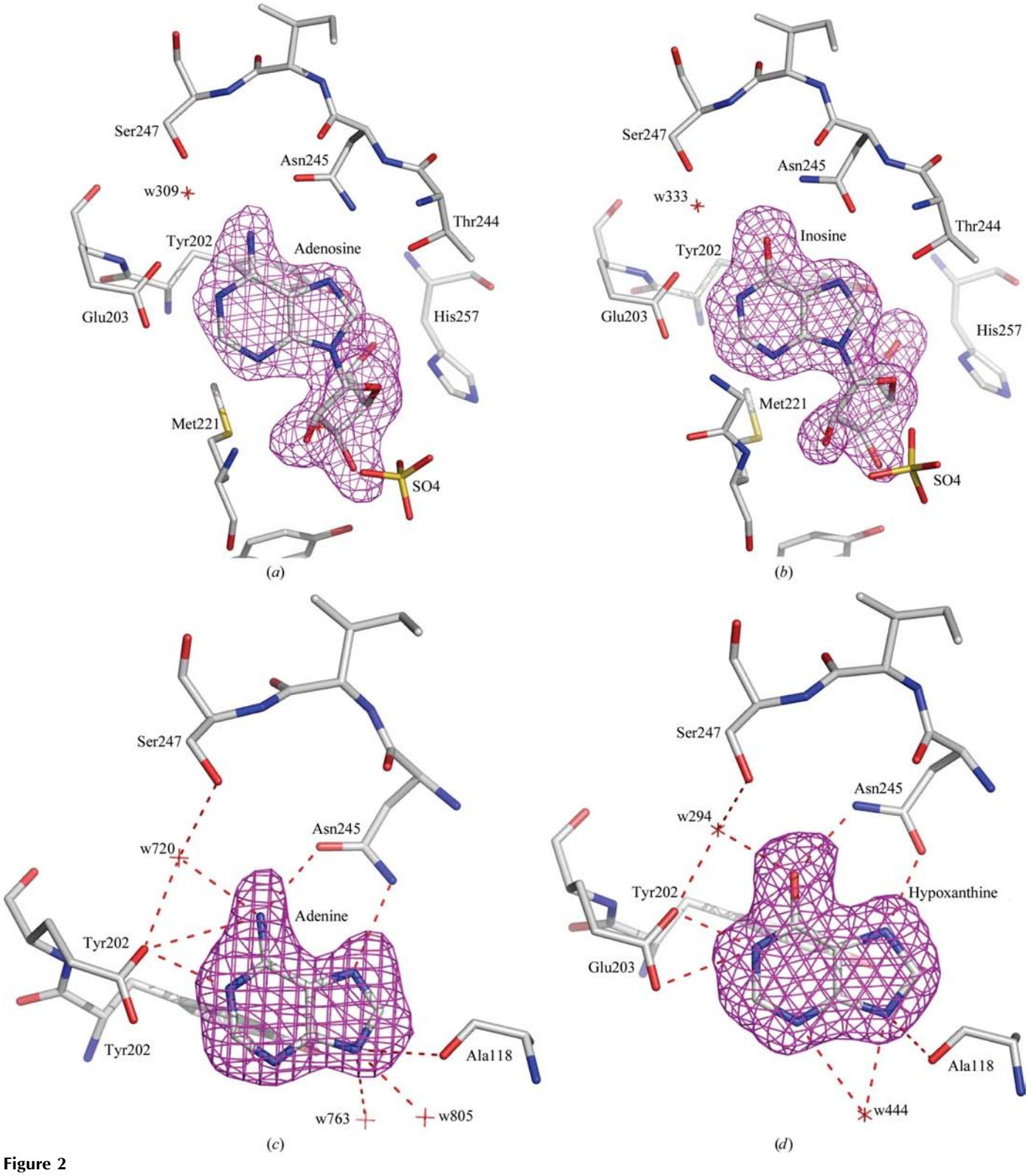

Figure 2
Standard $|F_o| - |F_c|$ OMIT maps contoured at 3σ showing the bound ligands in the active site of the *C* subunit of SmPNP. (*a*) Adenosine, (*b*) inosine, (*c*) adenine (*B* subunit) and (*d*) hypoxanthine.

bond between $N^{\delta 2}$ and the 6-oxo group is more compatible with the reverse reaction, in which N(7) of the hypoxanthine substrate could indeed be protonated owing to the absence of the ribose moiety and the consequent mixture of both N(7)—H and N(9)—H tautomeric forms (Koellner *et al.*, 1997). Under these circumstances, N(7) of hypoxanthine could donate a hydrogen bond to $O^{\delta 1}$ (Fig. 2*d*).

Adenine presents an essentially identical binding mode to that observed for the base in adenosine (Fig. 2*c*). However, adenine is not a measurable inhibitor of inosine phosphorolysis (data not shown), suggesting that the ribose moiety contributes significantly to binding. This is consistent with the fact that adenine is only observed bound to the *B* subunit and, unlike adenosine, is unable to induce the gate movement, which is mediated by contacts with the sugar.

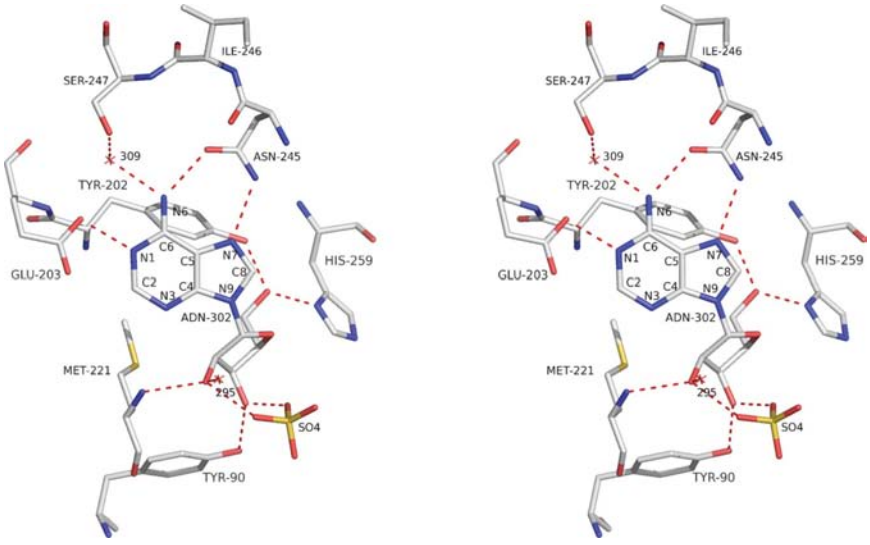

Figure 3
Stereo image of the hydrogen-bonding interactions made by adenosine bound to the active site of the *C* subunit. The adenosine molecule forms ten hydrogen bonds in total.

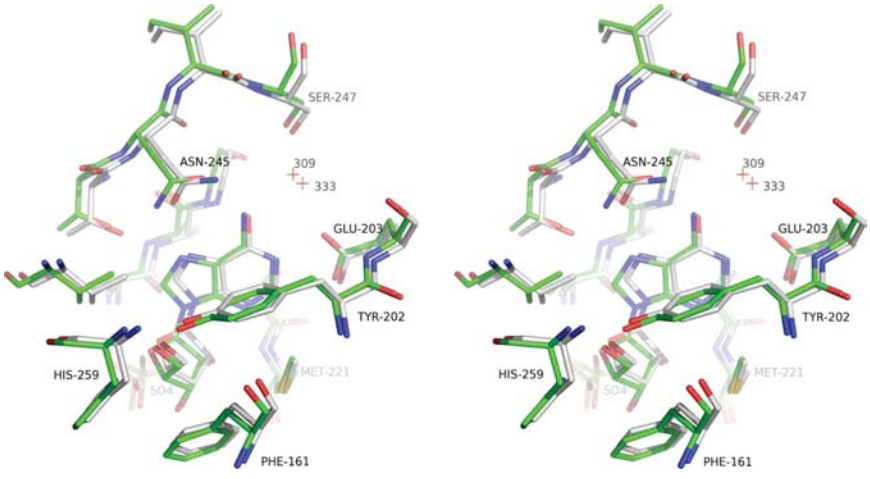

Figure 4
Stereoview superposition of the SmPNP active site in the complexes with adenosine (green) and inosine (white). In order to accomodate adenosine, the side chain of Asn245 is flipped in comparison with the inosine complex. Water 309 in the adenosine complex occupies a similar position to water 333 in the inosine complex.

It is known that the majority of LMW PNPs, including the human enzyme, bind adenosine only extremely weakly or not at all (Stoeckler *et al.*, 1997; Zimmerman *et al.*, 1971). As mentioned above, one exception is the PNP from *Cellulomonas* sp. (Tebbe *et al.*, 1999), for which adenosine presents a K_i of $\sim$160 μM, a feature that distinguishes it from the two main classes of PNPs. In this case adenosine behaves as an inhibitor rather than a substrate. The lack of catalytic activity is attributed to the absence of an H atom on N(1), which prevents the stabilization of the negatively charged intermediate during catalysis; this proposal was based on the catalytic mechanism described by Tebbe *et al.* (1999) and is strongly supported by recent evidence (Wielgus-Kutrowska & Bzowska, 2006). These authors further suggested that the ability of the *Cellulomonas* enzyme to bind adenosine at all is a consequence of the indirect interaction observed between the exocyclic substituent at C(6) and Asn246 (Asn245 in SmPNP). In the complex with 8-iodoguanine this interaction is mediated by a water molecule rather than being made directly as normally observed. The intervening water molecule could act either as a hydrogen-bond donor to a 6-oxo group or as an acceptor of a hydrogen bond from a 6-amino group, thus permitting the binding of different nucleosides including adenosine. However, this proposal remains speculative given that the structure of such an adenosine complex has yet to be reported.

On the other hand, in the current report we demonstrate that adenosine is observed to bind in a very similar fashion to that seen for inosine, including direct hydrogen bonds to Asn245 with no intervening water molecule (Fig. 4). This implies that the water-mediated interaction described for *Cellulomonas* cannot represent a generic explanation for adenosine binding as proposed previously (Tebbe *et al.*, 1999).

Examination of the details of the binding site shows that the presence of a serine at position 247 may provide at least part of the explanation for adenosine binding. As can be seen in Fig. 2(*a*), Ser247 anchors an additional water molecule within the active site (w309 in the *C* subunit of the adenosine complex) which forms a strong hydrogen bond to the 6-amino group of the adenine ring. Indeed, this water molecule is observed in all crystal structures of SmPNP in the presence or absence of bound ligands and therefore appears to be an intrinsic component of the active site, being part of a network which also involves Glu203. In two of the subunits (*B* and *C*) the temperature factor of this water is significantly lower

than the mean value (24.6 compared with 42.2 Å^2), whilst for subunit *A* it is somewhat higher (55.7 Å^2). Variation from one subunit to another is a recurrent theme in many of the PNP structures described to date. In contrast, w309 is not observed in any of the reported structures of the bovine or human enzymes owing to substitution of the serine by a valine (Val245). The valine side chain is incompatible with the presence of this water molecule, resulting in part of the hydrogen-bonding potential of the 6-amino group remaining unsatisfied, disfavouring adenosine binding (Fig. 5).

Examination of a nonredundant sequence alignment of 293 PNPs shows that serine and valine are relatively rare at position 247, being present in only 2.7 and 7.2% of sequences, respectively. The most commonly observed amino acids are alanine (present in more than 50% of all sequences), cysteine and glycine. For example, *Cellulomonas* PNP (which also binds adenosine) has an alanine at the homologous position (Ala248) and also presents a water molecule bound to the 6-oxo group of 8-iodoguanine in an approximately equivalent position. In this case, the lack of the side-chain hydroxyl group leads to a slight displacement of the water molecule in order to bind to the main-chain NH group of the following residue (Ala249) and/or the side chain of the catalytic Glu204 (Fig. 5). A similar situation is seen in the case of *Mycobacterium tuberculosis* (Shi *et al.*, 2001) and *Thermotoga maritima* PNPs (PDB code 1vmk; Joint Center for Structural Genomics, unpublished work), which have an alanine and cysteine, respectively, at the homologous position. We hypothesize that all such enzymes would be competent adenosine binders. Whatever the slight variations in the way in which this water is bound, it is clear that it is excluded by steric hindrance in those enzymes, such as human, that possess a valine at this position. For example, superposition of the SmPNP–adenosine complex with the bovine enzyme bound to inosine shows that the closest distance between the water molecule and the valine side chain would be of the order of 1.7 Å.

Although the importance of the bridging water molecule between the exocyclic group at the 6 position and Asn245 suggested by Tebbe *et al.* (1999) cannot be ruled out, it is clear that this is not essential in the case of SmPNP. Rather, we suggest that the ability to bind w309 may instead be the common feature which unites those PNPs that are capable of binding 6-amino nucleosides.

A second feature common to the *Cellulomonas* and *Schistosoma* enzymes is the replacement of Phe200 in the human enzyme by tyrosine (Tyr202 in SmPNP). The presence of the hydroxyl group of the tyrosine leads to the formation of a hydrogen bond to O5′ of the ribose moiety, possibly aiding in further increasing the affinity for adenosine (Fig. 2).

What is most noteworthy, however, is the difference from the human enzyme, which suggests that adenosine analogues may be a potential alternative for the design of specific inhibitors. Indeed, one adenosine analogue, tubercidin (9-deaza adenosine), has been used in the past as an experimental chemotherapeutic agent against schistosomiasis in animals (el Kouni & Cha, 1987; el Kouni *et al.*, 1987; Ross & Jaffe, 1972). In *in vitro* studies, tubercidin at a concentration at 0.1 µ*M* resulted in the separation of paired adult worms as well as alteration of patterns of muscular activity and inhibition of egg-laying. Unfortunately, it also resulted in severe host toxicity (Ross & Jaffe, 1972). Nevertheless, this study demonstrates the possibility of the use of adenosine analogues in treatment of schistosomiasis and we add to this a structural framework for their improvement.

Tremendous advances have been made towards the development of PNP inhibitors for the treatment of debili-

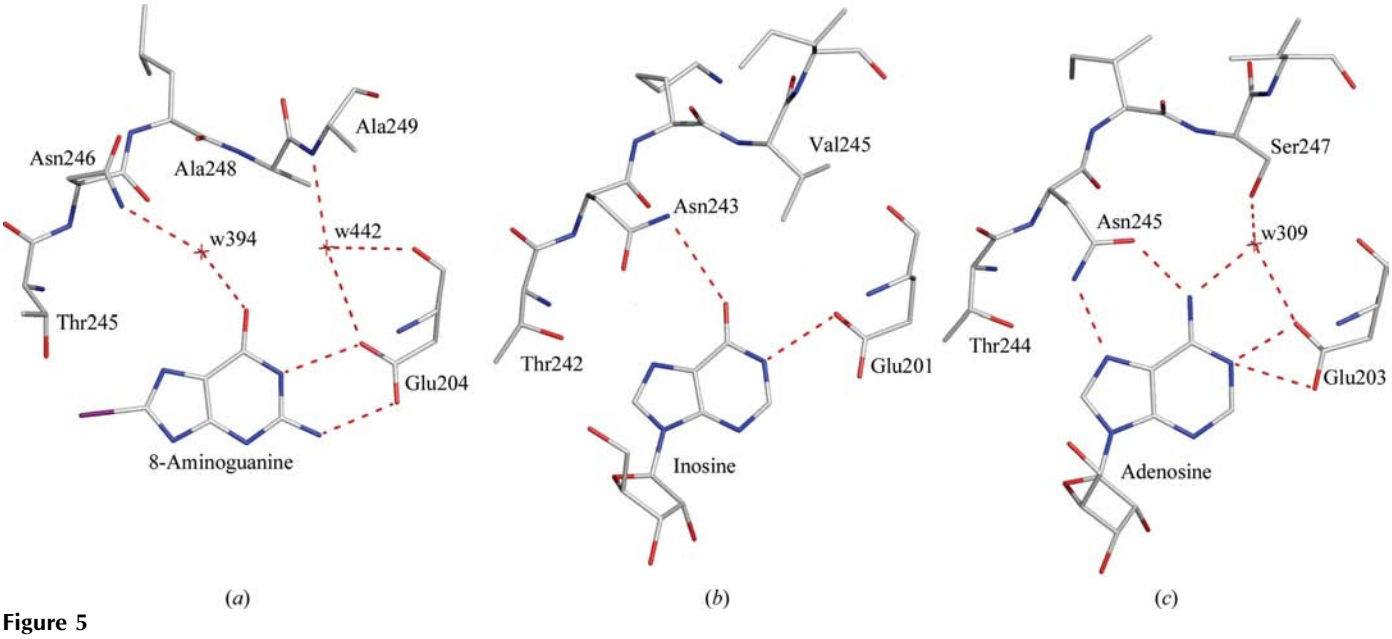

Figure 5
The importance of w309 in the adenosine complex and its analogue (w394) in the crystal structure of the complex of *Cellulomonas sp.* PNP with 8-aminoguanine. The presence of this water in the bovine and human enzymes is sterically prohibited by the side chain of Val245. (*a*) *Cellulomonas* sp. PNP bound to 8-aminoguanine, (*b*) bovine PNP bound to inosine and (*c*) *S. mansoni* PNP bound to adenosine.

tating diseases caused by human parasites, notably the immucillins in the treatment of malaria (Madrid *et al.*, 2008; Ting *et al.*, 2005; Schramm, 2004; Shi *et al.*, 2004; Kicska *et al.*, 2002). The discovery of the unexpected binding of adenosine to SmPNP suggests that compounds bearing an adenine base would be expected to show a degree of selectivity towards the schistosome enzyme compared with its human counterpart. This could be subsequently exploited during further rounds of optimization. Our results emphasize the importance of taking into consideration indirect solvent-mediated interactions when attempting to exploit structural information for inhibitor design.

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