Design of an adenosine phosphorylase by active-site modification of murine purine nucleoside phosphorylase

Enzyme kinetics and molecular dynamics simulation of Asn-243 and Lys-244 substitutions of purine nucleoside phosphorylase

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Our objective was to alter the substrate specificity of purine nucleoside phosphorylase such that it would catalyse the phosphorolysis of 6-aminopurine nucleosides. We modified both Asn-243 and Lys-244 in order to promote the acceptance of the C6-amino group of adenosine. The Asn-243-Asp substitution resulted in an 8-fold increase in K_m for inosine from 58 to 484 μM and a 1000-fold decrease in k_{cat}/K_m. The Asn-243-Asp construct catalysed the phosphorolysis of adenosine with a K_m of 45 μM and a k_{cat}/K_m 8-fold that with inosine. The Lys-244-Gln construct showed only marginal reduction in k_{cat}/K_m, 83% of wild type, but had no activity with adenosine. The Asn-243-Asp;Lys-244-Gln construct had a 14-fold increase in K_m with inosine and 7-fold decrease in k_{cat}/K_m as compared to wild type. This double substitution catalysed the phosphorolysis of adenosine with a K_m of 42 μM and a k_{cat}/K_m twice that of the single Asn-243-Asp substitution. Molecular dynamics simulation of the engineered proteins with adenine as substrate revealed favourable hydrogen bond distances between N7 of the purine ring and the Asp-243 carboxylate at 2.93 and 2.88 Å, for Asn-243-Asp and the Asn-243-Asp;Lys-244-Gln constructs respectively. Simulation also supported a favourable hydrogen bond distance between the purine C6-amino group and Asp-243 at 2.83 and 2.88 Å for each construct respectively. The Asn-243-Thr substitution did not yield activity with adenosine and simulation gave unfavourable hydrogen bond distances between Thr-243 and both the C6-amino group and N7 of the purine ring. The substitutions were not in the region of phosphate binding and the apparent $S_{0.5}$ for phosphate with wild type and the Asn-243-Asp enzymes were 1.35 ± 0.01 and 1.84 ± 0.06 mM, respectively. Both proteins exhibited positive co-operativity with phosphate giving Hill coefficients of 7.9 and 3.8 respectively.

Key words: molecular dynamics, protein engineering.

INTRODUCTION

Considerable effort is being expended on the design of drugs based upon structural information on the active site of enzymes [1,2]. Tertiary structure information is also being utilized in the modification of proteins thereby conferring novel functions or specificities. One such example is the specific modification of the thermostable Taq DNA polymerase to accept both 2′-deoxy- and 2′,3′-dideoxy-nucleoside triphosphates with equivalent affinity and rate [3], a modification which is of great value in DNA sequencing methodologies. Other such alterations in an enzyme may facilitate the conversion of certain otherwise inactive substrate analogues to products which can be further metabolized to active species.

Mammalian cells are devoid of an activity which phosphorolytically converts adenosine or deoxyadenosine to adenine and ribose phosphate [4,5]. In contrast there is a ubiquitously expressed purine nucleoside phosphorylase (PNP) which catalyses the phosphorolysis of inosine and guanosine and their deoxyribonucleoside counterparts [6,7]. A deficiency of this activity results in selective T cell immunodeficiency [8]. In spite of the functional deficit of an adenosine phosphorylase, mammalian cells do form adenine from the phosphorolytic cleavage of methylthioadenosine in the pathway of polyamine catabolism [9]. Adenine can be reutilized for nucleotide synthesis in the reaction catalysed by adenine phosphoribosyltransferase [10]. Although adenosine and deoxyadenosine are not cleaved to the free base, they may be phosphorylated to their corresponding nucleotides via respective kinases or deaminated via a common route catalysed by adenosine deaminase (ADA). The deficiency of ADA results in severe combined immunodeficiency [11] and ADA enzyme therapy has proven to be a useful and successful mode of treatment for ADA-deficient patients [12]; gene therapy approaches are actively used in trials [13].

Key interactions between specific residues of PNP and the natural substrates are apparent in the crystal structures of the protein which have been resolved at 2.75 Å for the human erythrocyte enzyme [14] and 2.15 Å [15] and 2.0 Å [16] for the bovine spleen enzyme. The carboxylate side chain of Glu-201 facilitates substrate binding through a bidentate interaction with both the N1 and C2-amino substituent of the guanine ring [14–18]. The ϵ-amino group of Lys-244 [14,17] and the carboxamido group of Asn-243 may both form hydrogen bonds with the C6-oxo moiety and N7 of the purine ring [14–18]. This latter interaction is believed to be catalytically important in stabilizing the proposed oxocarbenium transition state [15–20]. The association of the ϵ-amino group of Lys-244 with the C6-oxo substituent in the erythrocyte solution is not supported by the

Abbreviations used: PNP, purine nucleoside phosphorylase; ADA, adenosine deaminase.

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bovine spleen structure which revealed the Asn-243-Lys-244 peptide bond to be in the *cis* configuration [15]. In this orientation the side chain of lysine does not directly interact with the purine base.

In the present work we explore the design of a mammalian adenosine phosphorylase from PNP. There is potential that such an activity would be a useful alternative for enzyme or gene therapy in ADA deficiency, in target drug activation or in the synthesis of novel nucleoside analogues. We have previously cloned and sequenced murine PNP cDNA [21] and described the molecular basis of four naturally occurring [22] and three mutant alleles [23]. In preliminary studies we demonstrated the Asn-243-Asp substitution to confer the ability to catalyse the phosphorolysis of adenosine [24], as have others for the human enzyme [25]. In this report we examine the consequence of substitutions to Asn-243 and Lys-244 on the substrate specificity and kinetic parameters of PNP. The consequences of these changes upon the interactions of active site residues with the substrate, adenine, was examined by molecular dynamics simulation.

MATERIALS AND METHODS

Site-directed mutagenesis

We have previously reported the subcloning, expression and purification of the mouse PNP cDNA Np^b allele using the bacterial expression vector pMAL-c2 (New England BioLabs) [26]. The PNP–Asn-243-Asp construct was made from Np^b cDNA subcloned in Bluescript (Stratagene) using the procedure described by Ito et al. [27]. PCR conditions were as previously described using Pfu DNA polymerase (Stratagene); 2.5 units/0.050 ml was used throughout [26]. Two primer pairs were used in separate reactions: (a) T7 and Mut727 (GACAACCTTGTCCGTAATG, position 738–720) which contains a single substitution at nucleotide 727 resulting in the codon change; and (b) T3 and Mut-1 (GTGACTCAATGGAGAACG, position −8 to 10) which contains a single nucleotide substitution at position −1 abolishing the *Nco*I site. A 1/1000-fold dilution of reaction products (a) and (b) were used as templates in a second amplification using the T7 and T3 primers. The resulting products were digested with *Nco*I and *Hin*dIII and ligated into pTrc99a for preliminary expression and sequencing. The product was subsequently amplified by PCR with Pfu DNA polymerase (Stratagene) using the primers 5′(dTCGGTGA*ATT*CATGGAGAACG) at −11 to +10 and 3′(dAG*A*AGC*TT*AGCACTCCATTGCAGG) at 894 to 871, which contain *Eco*RI and *Hin*dIII sites, respectively, and was subcloned into pMAL-c2 as previously described [26].

The remainder of the substituted proteins were generated by a polymerase chain reaction-based mutagenesis methodology using the Np coding sequence in Bluescript and the combination of one mutagenic primer and a wild-type reverse primer, 720-GAGTGAGAAACCAAAGACACGGAG-697. The mutagenic primers were for: Lys-244-Gln, 721-ATTACGAAC*C*AGGTTGTCAT-740; Asn-243-Asp; Lys-244-Gln; 721-ATTACG*G*AC*C*AGGTTGTCAT-740; Asn-243-Thr, 721-ATTACGA*C*CAAGGTTGTCAT-749; Asn-243-Thr; Lys-244-Gln, 721-ATTACGA*CC*AGGTTGTCAT-740. The oppositely oriented mutagenic and wild-type primer pairs have no overlapping sequence but meet at their 5′ ends. PCR amplification utilized one of the mutagenic primers, the wild-type primer, Vent DNA polymerase (New England Biolabs), template, dNTPs and buffer according to supplier's specifications. The mixture was subjected to 30 cycles at 95 °C for 1 min, 54 °C for 1 min, 72 °C for 3 min with an initial stage of 95 °C for 5 min and a final extension for 12 min at 72 °C. All constructs were verified by sequence analysis of the entire coding region. The final cleaved products have four extra amino acids at the N terminus, Ile-Ser-Glu-Phe.

Expression and purification of wild-type and engineered PNP

The PNP–maltose binding fusion proteins were purified by amylose affinity column, cleaved with Factor Xa, and passed a second time over the affinity column as was previously described [26]. A further pass over the amylose affinity column yielded homogeneous products as monitored by SDS/12% PAGE. Maltose binding protein was from New England BioLabs and molecular weight standards were from BioRad Laboratories.

Enzyme assay and kinetic experiments

PNP was assayed in a real-time spectrophotometric assay by following the conversion of inosine to hypoxanthine in the presence of 30 mM phosphate, pH 7.0 at 37 °C, unless otherwise specified in a coupled reaction measured at 512 nm [28]. The reaction consisted of 1 mM 2,4,6-tribromo-3-hydroxybenzoic acid (Boehringer–Mannheim, U.K.), 0.1 mM 4-amino-antipyrene (Sigma), xanthine oxidase 0.025 unit/ml, uricase, 0.00325 unit/ml and peroxidase 0.002 unit/ml (all enzymes from ICN Pharmaceuticals). The concentrations of PNP and derivative constructs in the assay were approx. 0.08 to 5.9 μg/ml.

When adenine-containing nucleosides were used as substrates, the concentration of xanthine oxidase was increased 5-fold to facilitate the formation of 2,8-dihydroxyadenine from adenine. Extinction coefficients were determined empirically over a range of concentrations and were 15.4 or 75.8 nmol of substrate converted per unit absorbance change, for hypoxanthine or adenine as products respectively. Nucleoside phosphorolysis was proven to be rate limiting for all conditions by comparison to the rate with either adenine or hypoxanthine.

Initial rates in the kinetic experiments were analysed by a weighted nonlinear least-squares curve-fitting program [29] and co-operativity was examined by fit to the Hill equation using the Marquardt–Levinberg algorithm and programs of Brooks [30].

Molecular modelling

Crystallographic coordinates of the X-ray structure of PNP reported to a resolution of 3.2 Å were obtained from Brookhaven Protein Data Bank [14]. Modelling began with this structure having guanine bound to the enzyme (PDB reference 1ULB). Hydrogens were first explicitly added to the raw data using idealized bond lengths and valence angles as determined by the consistent valence force field (CVFF) of the Insight II program [31]. The substrate was changed to adenine, using the Biopolymer module of Insight II and bulk water was added to the molecule in a 10 Å layer using the Soak command of the program.

All molecular modelling was done on a Silicon Graphics MIPS R10000 Indigo 2 workstation using CVFF and the Discover3 molecular dynamics software module of the Insight II molecular modelling graphical interface (Molecular Simulations Inc.). To prepare the molecule for dynamics simulation the native protein structure with adenine as substrate underwent 10000 steps each of conjugate gradient and steepest descent minimization, the criteria being a derivative of less than 0.1 Å. This minimized structure then underwent a 100000 step dynamics simulation using the Discover3 dynamics software with a step size of 1 fs (total time 0.1 ns). The calculations were done at 298 K, pH 6.5, with all trajectories calculated using an atom-based non-bonded parameter; a 12 Å non-bonded cut-off distance was used at a dielectric constant of 2. This structure was the basis for all other

mutational studies. Further dynamics simulations were specifically performed within a 15 Å radius of the active site, with the remainder of the protein being free to accommodate structural changes that occurred within this region. The desired residue substitutions were made separately to this molecule using the Biopolymer module of the Insight II program. A 10000 step minimization and 100000 step dynamics simulation were then run on each molecule in the same manner as above. A structure was taken every 5000 steps during the dynamics simulation. Upon completion of the simulation, these trajectories were loaded and graphed using the Analysis module of Insight II in order to identify which structures had attained energy equilibrium for the molecule. Using the trajectory-conformation-average command the conformations that constituted the stable state were used to create an average protein model. The averaged final structure was used to determine bond distances and enzyme–substrate interactions. This complete process was carried out for all five of the altered proteins that were expressed and characterized in this study.

RESULTS

Based on crystal structures of PNP we have sought to alter the substrate specificity such that adenine nucleosides might be preferred to the natural purine substrates inosine and guanosine. We examined the substitution of Asn-243 with Asp or Thr and Lys-244 with Gln in order to change the preference for the C6-oxo moiety of hypoxanthine or guanine to that of the C6-amino moiety of adenine. These constructs and their combinations in addition to the wild-type coding sequence were subcloned into the bacterial expression vector pMAL-c2 and sequenced in their entirety. The expressed fusion proteins were purified to homogeneity by affinity chromatography on an amylose affinity matrix.

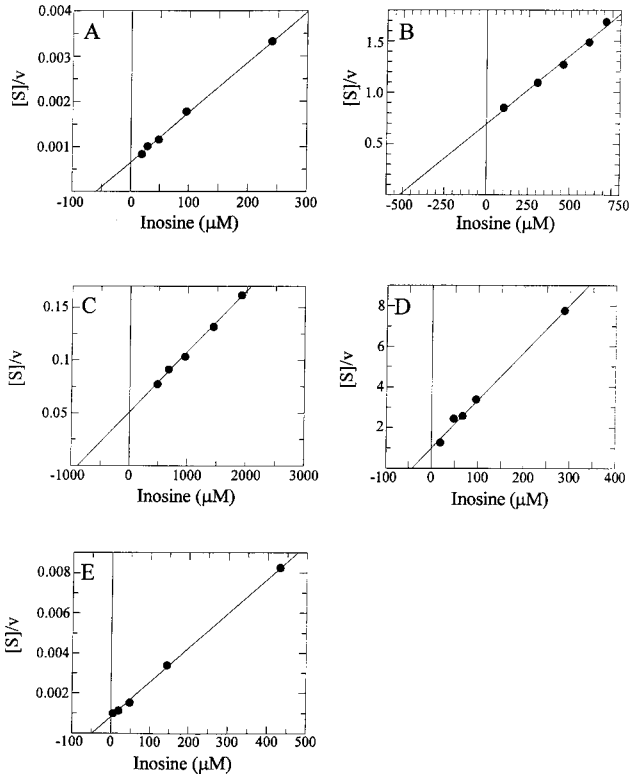

Figure 1 Hanes–Woolf plot of wild-type and mutant enzymes with inosine as variable substrate

Phosphate was constant at 30 mM for: (**A**) wild type; (**B**) Asn-243-Asp; (**C**) Asn-243-Asp; Lys-244-Gln; (**D**) Asn-243-Thr; (**E**) Lys-244-Gln.

Table 1 Kinetic constants for wild-type and engineered PNPs

Assays were conducted with variable concentration of nucleoside substrate as given in Figures 1 and 2 and fixed concentration of phosphate, 30 mM, pH 7.0. N.D., not determined; N.D.A., no detectable activity with variable substrate (estimate < 25 nmol/min · mg protein).

Enzyme	V_{max} (nmol product/ min · mg protein)	K_m (μM)	k_{cat} (s^{-1})	(k_{cat}/K_m) ($s^{-1} \cdot M^{-1}$)
(A) Substrate: inosine				
Wild type	88673 ± 1016	58.0 ± 2.2	47.3	815×10^3
Asn-243-Asp	725.0 ± 45	484 ± 62	0.386	798
Asn-243-Asp; Lys-244-Gln	17007 ± 486	823 ± 53	9.10	11.1×10^3
Asn-243-Thr	45.7 ± 2.2	54.0 ± 9.8	0.024	445
Asn-243-Thr; Lys-244-Gln	< 10	N.D.	N.D.	N.D.
Lys-244-Gln	58196 ± 962	45.9 ± 3.5	31.0	676×10^3
(B) Substrate: adenosine				
Wild type	< 20	N.D.	N.D.	N.D.
Asn-243-Asp	529 ± 18	45.4 ± 5.5	0.282	6210
Asn-243-Asp; Lys-244-Gln	1085 ± 45	41.7 ± 8.0	0.579	13800
Asn-243-Thr	N.D.A	N.D.	N.D.	N.D.
Asn-243-Thr; Lys-244-Gln	N.D.A.	N.D.	N.D.	N.D.
Lys-244-Gln	N.D.A.	N.D.	N.D.	N.D.

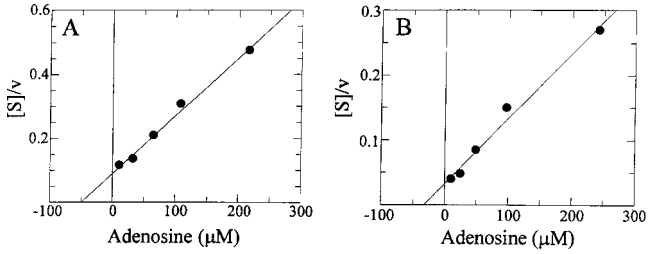

Figure 2 Hanes–Woolf plot of mutant constructs with adenosine as variable substrate

Phosphate was constant at 30 mM for: (**A**) Asn-243-Asp and (**B**) Asn-243-Asp; Lys-244-Gln.

The wild-type murine protein had kinetic parameters comparable to those of previously characterized mammalian purine nucleoside phosphorylases [5–7,32,33] and had no measurable activity with adenosine. Hanes–Woolf plots and the kinetic parameters for wild-type PNP and the five constructs are given in Figure 1 and Table 1. The Asn-243-Asp and Asn-243-Asp; Lys-244-Gln constructs showed a desirable decrease in affinity for inosine with 8- and 14-fold increases in K_m, respectively (Table 1). The catalytic efficiency was also substantially reduced. The single Lys-244-Gln substitution showed only a marginal reduction in catalytic efficiency to 83% of wild type and an apparent decrease in K_m for inosine. There was a marked loss in activity with inosine for the Asn-243-Thr substitution and essentially no measurable activity with the Asn-243-Thr; Lys-244-Gln substitution.

Of the mutant constructs, the Asn-243-Asp and Asn-243-Asp; Lys-244-Gln proteins catalysed the phosphorolysis of adenosine with K_ms of 45 and 42 μM, respectively (Table 1, Figure 2).

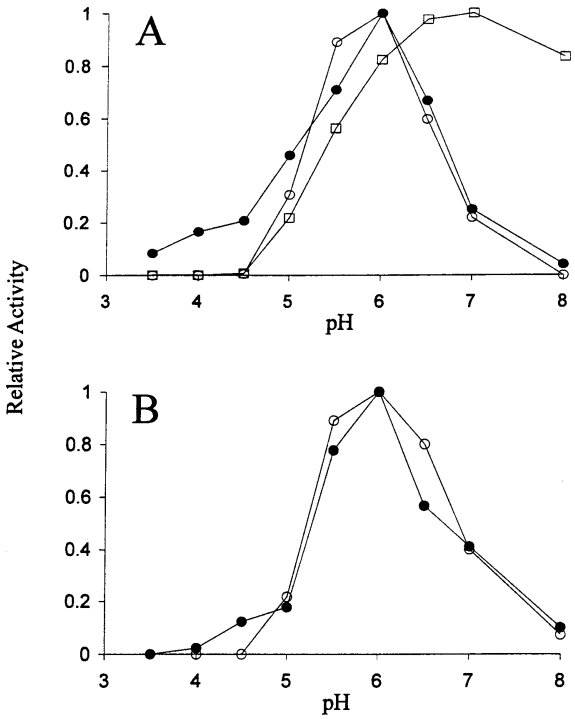

Figure 3 Relative activity as a function of pH for purine nucleoside phosphorylase wild-type and engineered enzymes

Results are expressed as a fraction of the optimal activity in the presence of 30 mM phosphate for: (**A**) Wild type (□) with 40 μM inosine, Asn-243-Asp (○) with 400 μM inosine and Asn-243-Asp;Lys-244-Gln (●) with 800 μM inosine, and (**B**) Asn-243-Asp (○) and Asn-243-Asp;Lys-244-Gln (●) with 40 μM adenosine.

The catalytic efficiencies for these proteins with adenosine were reduced 130- and 60-fold respectively, as compared to wild-type enzyme with inosine. These two constructs and the wild-type enzyme were subjected to further kinetic analyses. Both constructs exhibited a sharp pH optimum at 6.0, in contrast to the wild-type enzyme which has a broader optimum between pH 6.5–7.5 (Figure 3). At pH 8 the wild-type enzyme retained 80% of its activity whereas the two constructs had less than 5% of maximal activity. The K_m, k_{cat} and k_{cat}/K_m with inosine and adenosine were also examined at pH 6 and 7 for wild-type and mutant enzymes respectively (Table 2). In order to assess stability, enzymes were adjusted to extreme pHs as in assay conditions, for 10 min at 37 °C, followed by assay at pH 7. Wild-type and the Asn-243-Asp proteins showed no detectable loss in activity, $< 2\%$, by exposure to pH 5 or 8. The Asn-243-Asp;Lys-244-Gln mutant showed a 5% loss at pH 8 and an 11% loss at pH 5. Significant and variable loss for all proteins was encountered by exposure to pH 4. Thus the differences in kinetic properties observed between constructs at pH 6 and 7 (Figure 3 and Table 2) were not due to altered stability.

The change in purine base specificity for the two constructs having activity with adenosine was further probed by examining their sensitivity to the non-cleavable inosine and adenosine C–C glycoside analogues, formycin B and formycin A, respectively (Table 3). The wild-type enzyme had a 2.4-fold greater K_i with the adenosine analogue as compared to the inosine analogue. In contrast, the K_i was 5-fold lower for formycin A than formycin B with the Asn-243-Asp construct and greater than 10-fold lower with the Asn-234-Asp;Lys-244-Gln construct, clearly demonstrating their enhanced sensitivity toward inhibition by the analogue having the C6-amino group as opposed to the C6-oxygen moiety.

The kinetic properties of the wild-type and the Asn-243-Asp enzymes were also examined with the co-substrate, phosphate.

Table 3 Inhibition of wild type and engineered PNP by formycin A and B

Assays were conducted with variable concentrations of nucleoside substrate and inhibitor as given below at fixed concentration of phosphate, 30 mM, pH 7.0. N.D., not determined; Wild type, formycin B at: 0, 40, 80, 120, 200, 300 μM vs. inosine at: 25, 50, 75, 100, 125 μM; Asn-243-Asp: formycin B at: 0, 50, 100, 200, 300, 500 μM vs. adenosine at: 50, 100, 150, 200, 300 μM; Wild type: formycin A at: 0, 50, 100, 200, 300, 400 μM vs. inosine at: 25, 50, 75, 100, 125 μM; Asn-243-Asp: formycin A at 0, 100, 200, 300, 400, 500 μM vs. adenosine at: 50, 100, 150, 200, 300 μM; Asn-243-Asp;Lys-244-Gln: formycin B at: 0, 100, 200, 300, 400 μM vs. inosine at: 400, 800, 1200 μM, *or* vs. adenosine at: 20, 40, 100 μM; Asn-243-Asp;Lys-244-Gln: formycin A at: 0, 100, 200, 300, 400 μM vs. inosine at: 400, 800, 1200 μM; *or* formycin A at: 25, 50, 75, 100 μM vs. adenosine at: 20, 40, 100 μM.

Enzyme	Formycin A K_i (μM)	Formycin B K_i (μM)
(A) Substrate: inosine		
Wild type	667 ± 68	279 ± 19
Asn-243-Asp	N.D.	N.D.
Asn-243-Asp;Lys-244-Gln	114 ± 28	339 ± 59
(B) Substrate: adenosine		
Wild type	N.D.	N.D.
Asn-243-Asp	143 ± 23	670 ± 111
Asn-243-Asp;Lys-244-Gln	68.5 ± 6.4	705 ± 166

Table 2 Kinetic constants for wild-type and engineered PNPs as a function of pH

Assays were conducted in 30 mM phosphate, pH 6.0 or pH 7.0.

		pH 6.0			pH 7.0		
Enzyme	Substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1} \cdot M^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1} \cdot M^{-1}$)
Wild type	Inosine	32 ± 2	38.9 ± 0.9	1217×10^3	58 ± 2	47.3 ± 0.5	815×10^3
Asn-243-Asp	Inosine	691 ± 120	1.76 ± 0.17	2.55×10^3	484 ± 62	0.386 ± 0.024	0.80×10^3
	Adenosine	45 ± 9	0.71 ± 0.05	15.8×10^3	45 ± 5	0.282 ± 0.010	6.27×10^3
Asn-243-Asp;Lys-244-Gln	Inosine	729 ± 206	36.4 ± 4.8	49.9×10^3	823 ± 6	9.10 ± 0.26	11.1×10^3
	Adenosine	28 ± 4	1.41 ± 0.06	50.4×10^3	42 ± 8	0.579 ± 0.024	13.8×10^3

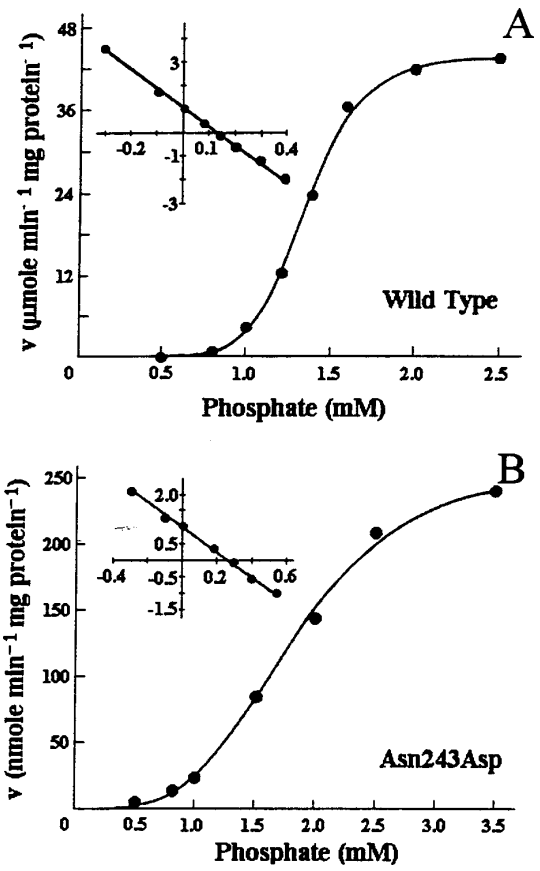

Figure 4 Initial velocity of PNP and Asn-243-Asp with variable concentration of phosphate

V vs. [*S*] plot at fixed concentration of nucleoside, 400 μM inosine for wild type or 400 μM adenosine for Asn-243-Asp. Hill plots of data given as inserts, as log $(V_{max} - v)/v$ vs. log [phosphate].

Both enzymes exhibited sigmoidal kinetics (Figure 4) characteristic of positive cooperativity with the binding of the substrate phosphate. The data was analysed by fit to the Hill equation and gave Hill coefficients of 7.95 ± 0.55 and 3.79 ± 0.35 for wild type and Asn-243-Asp, respectively (Figure 4). The $S_{0.5}$ with phosphate did not substantially differ between these proteins, 1.35 ± 0.01 and 1.84 ± 0.06 mM for wild type and Asn-243-Asp, respectively.

Each of the five variant proteins was subjected to molecular dynamics simulation with adenine in the active site in order to examine the consequence of the substituted residues upon their interaction with the novel substrate (Figure 5). Atomic distances are shown for potential hydrogen bond interactions between the key residues and the purine substrate. A summary of the relevant changes in bond distances is given in Table 4.

DISCUSSION

Our objective was to explore the possibility of modifying the substrate specificity of PNP in order that C6-amino substituted nucleosides such as adenosine might be preferred substrates. We therefore made substitutions to Asn-243 and Lys-244 in the mouse Np^b allele of PNP. The kinetic findings were interpreted in the context of molecular dynamics simulation of active site interactions using the human PNP crystallographic structure. The human and mouse PNP proteins share 84% identity and the active site residues for the human, mouse and bovine enzymes are strictly conserved.

Crystallographic analysis of PNP has provided evidence for the binding specificity and preference for the 6-oxopurine substrates, inosine and guanosine [14–16]. The X-ray data are consistent with hydrogen bond interaction between the N1 of the purine ring and the side chain carboxylic acid of Glu-201. In addition there is evidence for the C2-amino substituent of guanosine to be involved in hydrogen bond donation to Glu-201 [14], which is the apparent reason for the slightly greater affinity of the enzyme for guanosine as opposed to inosine. The C6 oxygen of the natural substrates, inosine or guanosine, accepts a hydrogen bond from Asn-243 [14,15,17,18] or is linked to Glu-201 through a water molecule [15,16]. Weaker hydrophobic interactions occur between the faces of the purine ring and several residues lying above and below the substrate [14–16]. There is both kinetic [19,20] and crystallographic evidence for N7 acting as an acceptor of the side chain amido-hydrogen of Asn-243 [14–16,18]. This interaction is not viewed as being essential for substrate binding but is thought to be required for transition state stabilization during phosphorolysis because of a preprotonation step of N7 with the resultant negative charge being distributed between N7 of the purine ring and the glycosidic N9.

The absence of detectable activity with adenosine and its analogues is presumably due to the inability to accept the C6-amino substituent of the purine ring. The substrate specificity is illustrated by the 660-fold greater K_i for adenine, 6.6 mM, than for hypoxanthine, 0.01 mM, with human PNP [4]. Of further relevance, the crystal structure for the *E. coli* enzyme, which accepts both 6-oxy- and 6-amino-purine substrates, has an active site Asp as the residue corresponding to Asn-243 [34]. We have examined the roles of Asn-243 and Lys-244 in substrate specificity by site-directed mutagenesis and kinetic analysis of the engineered proteins in comparison to the wild-type protein. The catalytic efficiency of recombinant wild-type PNP was within the typical range for many proteins [35] having a k_{cat}/K_m of 0.8×10^6 $s^{-1} \cdot M^{-1}$ (Table 1).

The mutation of Asn-243 to Thr resulted in loss of activity with inosine and no activity was detected with adenosine. Molecular modelling showed no evidence for a favourable interaction of the side chain of Thr with N7 of the purine ring (Figure 5C, Table 4). The inability of Thr to participate in hydrogen bonding with N7 of the purine ring and thereby stabilize the transition state structure would be consistent with the loss of activity. Similar kinetic findings have been obtained for substitution of Asn-243 by Ala or Ser for the human enzyme [25,36].

The role of Lys-244 has been controversial with the peptide bond between Asn-243 and Lys-244 being defined in the *trans* conformation for the human erythrocytic structure with guanine [14]. The calf spleen structure, however, provided evidence for the Asn-243-Lys-244 peptide bond to exist in the *cis* conformation, one consequence being that the ϵ-amino group of Lys-244 is not directly involved in base recognition in this solution [15]. This is reflected in the absence of hydrogen bonding interaction between the C6 substitutent of the purine ring and Lys-244 for both bovine spleen structures [15,16] (Table 4). A further consequence is that the bond distance for Asn-243 with N7 of the purine ring is also more favourable for the bovine spleen enzyme with either guanine, 2.91 Å [15] or inosine, 2.90 Å [16], than for the human erythrocyte enzyme, 3.43 Å [14].

We reasoned, however, that the Lys-244-Gln substitution might facilitate a favourable interaction of the carboxamide side

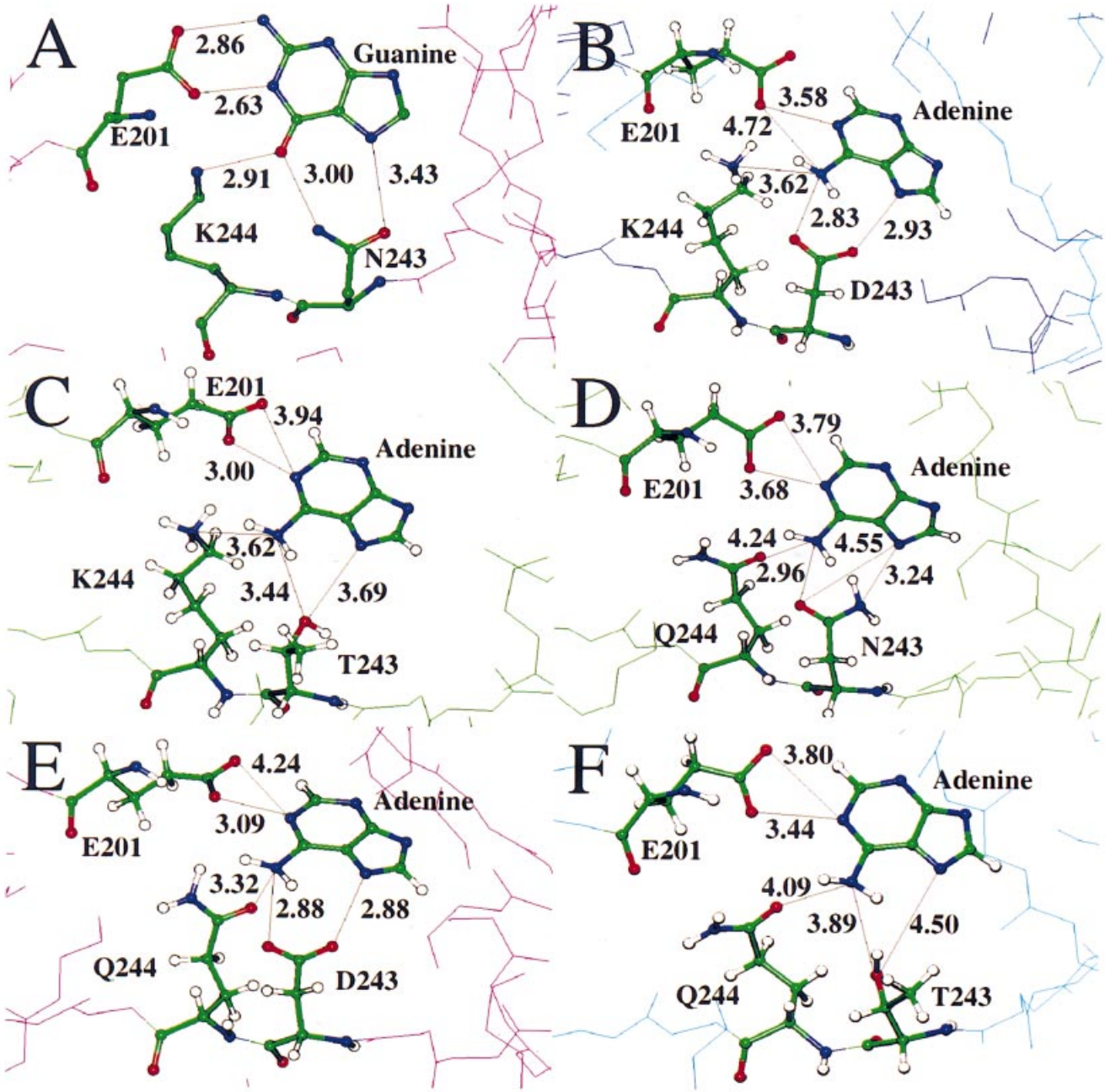

Figure 5 Molecular dynamics simulation of active site for PNP constructs with adenine as substrate as compared to the human erythrocyte PNP structures

(**A**) Human erythrocyte PNP with guanine. (**B**) Asn-243-Asp with adenine. (**C**) Asn-243-Thr with adenine. (**D**) Lys-244-Gln with adenine. (**E**) Asn-243-Asp;Lys-244-Gln with adenine. (**F**) Asn-243-Thr;Lys-244-Gln with adenine. The atomic colour code is used: carbon, green; nitrogen, blue; oxygen, red; hydrogen, white.

chain of glutamine with the C6-amino substituent of adenosine. Interestingly this single substitution resulted in a slight increase in affinity for inosine, with K_ms of 58 and 46 μM for wild type and Lys-244-Gln mutant, respectively (Table 1). The catalytic efficiency was only marginally reduced. There was, however, no detectable activity with adenosine as substrate (Table 1). Molecular dynamics simulation of the Lys-244-Gln with adenine as substrate revealed an unfavourable N7 Asn-243 side chain bond distance, 3.24 Å (Figure 5D, Table 4) consistent with the absence of activity with adenosine. The Lys-244-Ala substitution in the human enzyme also resulted in minimal change in both the K_m for inosine and the k_{cat} [36].

Two mutant constructs, Asn-243-Asp and Asn-243-Asp;Lys-244-Gln, catalysed the phosphorolysis of adenosine. Both of these enzymes showed a marked shift in pH optimum to pH 6.0 as compared to wild type, which has a broader and more basic optimum (Figure 3). The catalytic efficiency, k_{cat}/K_m, was also 2.5- and 3.8-fold greater at pH 6 than pH 7 for Asn-243-Asp and Asn-243-Asp;Lys-244-Gln, respectively with adenosine as substrate (Table 2). One possible consideration for the pH shift

Table 4 Potential hydrogen bond interactions between active site residues in PNP constructs and adenine as obtained by molecular dynamics simulations

O6 represents the exocyclic oxygen of guanine and hypoxanthine and N6 the exocyclic nitrogen of adenine.

Enzyme	Substrate	Atomic distances to amino acid side chain in Å			
		O6-Asn-243	O6-Lys-244	N1-Glu-201	N7-Asn-243
(A) Crystallographic					
Human erythrocyte [14]	Guanine	3.00	2.91	2.63	3.43
Bovine spleen [15]	Hypoxanthine	2.56	—	2.63	2.91
Bovine spleen [16]	Inosine	3.28	—	2.81	2.90
(B) Molecular simulation		N6-243	N6-244	N1-Glu-201	N7-243
Asn-243-Asp	Adenine	2.83	3.62	3.58	2.93
Asn-243-Thr	Adenine	3.44	3.62	3.00	3.69
Lys-244-Gln	Adenine	2.96	4.24	3.68	3.24
Asn-243-Asp; Lys-244-Gln	Adenine	2.88	3.32	3.09	2.88
Asn-243-Thr; Lys-244-Gln	Adenine	3.89	4.09	3.44	4.50

would be ionization differences between the two substrates, inosine, pK_a 8.9 versus adenosine pK_a 3.6 [37]. The difference between the wild-type enzyme and the Asn-243-Asp constructs, however, was not attributable to substrate ionization, as the engineered proteins exhibited the same pH shift with both inosine and adenosine (Figure 3). Thus if stabilization of the proposed transition state could be facilitated by a partially protonated Asp-243, the carboxylate could then interact as a hydrogen donor with the N7 of the purine ring and as a hydrogen acceptor with the C6-amino group of adenine. The argument has previously been advanced that the expected pK_a of a buried Asp is such that the carboxylate side chain can exist in a protonated form at pH 7 to a significant extent [38]. The simulated bond distances are favourable for hydrogen bonds between Asp-243 and the C6-amino and N7 of the purine ring for both the Asn-243-Asp and Asn-243-Asp; Lys-244-Gln constructs (Table 4, Figures 5B and 5E). The acidic pH optimum shift (Figure 3) is consistent with the participation of a protonated Asp-243 side chain in forming a hydrogen bond with N7 of the purine ring.

Favourable purine-N7 to Asp-243 hydrogen bond distances were obtained only for molecular dynamics simulation of the Asn-243-Asp and Asn-243-Asp; Lys-244-Gln constructs with adenine as substrate. The Asn-243-Thr substitution resulted in an unfavourable Thr-243 to N7 bond distance of 3.69 Å (Table 4). Similarly the Lys-244-Gln construct had an unfavourable Asn-243 to N7 bond distance of 3.24 Å. Neither Asn-243-Thr nor Lys-244-Gln proteins had activity with adenosine. Favourable hydrogen bond distances were obtained for the interaction of the C6-amino group of adenine with the Asn-243 substitution for both the Asn-243-Asp and Asn-243-Asp; Lys-244-Gln constructs, at 2.83 and 2.88 Å (Table 4). For these constructs, atomic distances were too great for possible hydrogen bond interactions between the side chains of Lys-244 or Gln-244 and the C6-amino substituent of adenine (Table 4). The Asn-243-Asp; Lys-244-Gln mutant had 2-fold greater k_{cat} and k_{cat}/K_m than the single Asn-243-Asp mutant at pH 7.0. In this instance molecular dynamics simulation predicts the doubly substituted construct to have a hydrogen bond length between Glu-201 and N1 of the purine ring of 3.09 Å versus 3.58 Å for the single mutant. This difference may have contributed in part to the greater catalytic efficiency of the double mutant.

There are kinetic differences between the recently reported human [25] and mouse Asn-243-Asp constructs (Table 1) which are not readily explicable. The kinetic parameters for the human and mouse wild-type enzymes with inosine were comparable for K_m, k_{cat} and k_{cat}/K_m. For example the K_ms for inosine were 45 and 58 μM for human [25] and mouse (Table 1), respectively. The Asn-243-Asp constructs both showed an increase in K_m for inosine as compared to wild type, 340 and 484 μM for human [25] and mouse (Table 1), respectively. The K_ms with adenosine, however, were divergent, being 740 and 45 μM for human and mouse respectively. Thus the human Asn-243-Asp construct had greater affinity for inosine than adenosine by 2-fold, whereas the mouse enzyme had a greater than 10-fold increase in affinity for adenosine as opposed to inosine (Table 1). There were also log fold differences in the k_{cat} between the human and mouse constructs. Although these differences are not readily explicable given the identity of the active site, further kinetic evidence has substantiated the improved specificity of the murine enzyme for the C6-amino group over the C6-oxygen moiety by the inhibition studies with formycin A and B. These studies showed the C6-amino analogue, formycin A, to have 4.6-fold and 10-fold lower K_is than the C6-oxygen analogue, formycin B, with the Asn-243-Asp and Asn-243-Asp; Lys-244-Gln constructs, respectively (Table 3). Thus both comparative substrate and inhibitor analyses demonstrated an increased affinity of the murine Asn-243-Asn constructs for C6-amino- over C6-oxygen-substituted purines.

The residues altered in this study should have no direct consequence upon phosphate binding in perspective of the crystallographic findings [14–16]. Wild-type and the Asn-243-Asp enzymes had comparable $S_{0.5}$ values with respect to phosphate and both exhibited positive co-operativity with this substrate (Figure 4), having Hill coefficients of 7.9 and 3.8, respectively. Significant nonlinearity has also been observed over a broad range of phosphate concentration with the calf spleen enzyme [39]. In these studies a Hill coefficient of 10.8 for phosphate was associated with the dissociation of the trimer to monomers [39]. A second sulphate-binding site was identified in the region of a loop formed by residues 32–42 of the human erythrocyte enzyme [14], but no equivalent site was apparent in the bovine spleen structure [15]. Structural changes in two regions, residues 32–36 and 56–69, are observed for the bovine enzyme that are specifically associated with phosphate binding [16]. Thus there is structural [14,16] and kinetic (Figure 4) [39] evidence for conformational change occurring with occupancy of the phosphate site.

In summary, we have by site-directed mutagenesis produced two engineered forms of murine PNP which are able to catalyse the phosphorolysis of adenosine. Both the Asn-243-Asp and Asn-243-Asp; Lys-244-Gln proteins show significant preference

for adenosine over inosine as substrate, and these constructs should prove useful in further exploring the role of a novel metabolic cycle in correcting the inherited deficiency of adenosine deaminase or metabolizing substrate analogues.

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