Purine Nucleoside Phosphorylase. 3. Reversal of Purine Base Specificity by Site-Directed Mutagenesis†

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ABSTRACT: Human purine nucleoside phosphorylase (PNP) is highly specific for 6-oxopurine nucleosides with a catalytic efficiency (k_{cat}/K_M) for inosine 350000-fold greater than for adenosine. Crystallographic studies identified Asn243 and Glu201 as the residues largely responsible for the substrate specificity. Results from mutagenesis studies demonstrated that the side chains for both residues were also essential for efficient catalysis [Erion, M. D., et al. (1997a) *Biochemistry 36*, 11725−11734]. Additional mechanistic studies predicted that Asn243 participated in catalysis by stabilizing the transition state structure through hydrogen bond donation to N7 of the purine base [Erion, M. D., et al. (1997b) *Biochemistry 36*, 11735−11748]. In an effort to alter the substrate specificity of human PNP, mutants of Asn243 and Glu201 were designed to reverse hydrogen bond donor and acceptor interactions with the purine base. Replacement of Asn243 with Asp, but not with other amino acids, led to a 5000-fold increase in k_{cat} for adenosine and a 4300-fold increase in overall catalytic efficiency. Furthermore, the Asn243Asp mutant showed a 2.4-fold preference for adenosine relative to inosine and a 800000-fold change in substrate specificity (k_{cat}/K_M) relative to wild-type PNP. The double mutant, Asn243Asp::Glu201Gln, exhibited a 190-fold increase in catalytic efficiency with adenosine relative to wild-type PNP, a 480-fold preference for adenosine relative to inosine, and a 1.7×10^8-fold change in preference for adenosine over inosine relative to wild-type PNP. The Asn243Asp mutant was also shown to synthesize 2,6-diaminopurine riboside with a catalytic efficiency (1.4×10^6 M^{-1} s^{-1}) on the same order of magnitude as wild-type PNP with its natural substrates hypoxanthine and guanine. The Asn243Asp mutants represent examples in which protein engineering significantly altered substrate specificity while maintaining high catalytic efficiency.

Purine nucleoside phosphorylase (PNP, EC 2.4.2.1) catalyzes the reversible phosphorolysis of purine nucleosides to generate the corresponding purine base and ribose 1-phosphate. Human PNP is highly specific for 6-oxopurine nucleosides and exhibits negligible activity for 6-aminopurine nucleosides. In some cases, no measurable phosphorolysis activity is reported for adenosine (Huennekens et al., 1956; Bzowska et al., 1990) whereas in other cases very low activity is found (e.g., human erythrocytic PNP, $K_M = 620$ μM and $k_{cat} = 7 \times 10^{-3}$ s^{-1}; Zimmerman et al., 1971). Adenosine synthesis has also been detected using mammalian PNPs, but with significantly lower catalytic efficiencies relative to hypoxanthine due to the high K_M for adenine (410 μM) and the large decrease in V_{max} (0.06% relative to hypoxanthine) (Zimmerman et al., 1971). Crystallographic studies of PNP−inhibitor complexes (Ealick et al., 1990) identified Asn243 and Glu201 as the active-site residues in the purine binding site responsible for the high substrate specificity based on the hydrogen bond donated to O6 by the Asn243 side-chain carboxamido group and the hydrogen bond accepted from N1 by the Glu201 side-chain carboxylate. Mutagenesis studies revealed that these residues were also important for catalysis (Erion et al., 1997a). In the previous paper, a substrate-assisted catalytic mechanism was proposed that utilizes the cosubstrate, phosphate, to stabilize an intermediate oxocarbenium ion (Erion et al., 1997b). In addition, the side-chain carboxamido group of Asn243 was postulated to aid catalysis by forming a hydrogen bond with N7 of the purine base in the transition state.

Interest in PNP, its substrate specificity, and its catalytic mechanism stems in large part from its potential as a catalyst for the enzymatic synthesis of nucleoside analogs (Krenitsky et al., 1986). Enzymes that catalyze the formation of the glycosidic bond are especially attractive for use in the synthesis of purine nucleoside analogs (Polastro et al., 1989), since synthesis of the glycosidic bond chemically often involves multistep processes that are plagued by low yields and contaminating regio- and stereochemical isomers. In addition, enzymatic synthesis of the glycosidic bond has the potential to decrease production costs and to dramatically shorten the synthetic route by eliminating the need for protecting groups. As a consequence, substantial efforts have

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[1] Abbreviations: PNP, purine nucleoside phosphorylase; DTT, dithiothreitol; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); PEG, polyethylene glycol; PMSF, phenylmethanesulfonylfluoride; MTA, methylthioadenosine; TS, transition state; Hyp, hypoxanthine; Ino, inosine; Guo, guanosine; Gua, guanine; Ado, adenosine, Ade, adenine.

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been made to use PNP in the large-scale synthesis of nucleosides. For example, bacterial PNP has been used to prepare 2,6-disubstituted purine nucleoside analogs (Krenitsky et al., 1981), 3-deazapurine nucleosides (Krenitsky et al., 1986), ribavirin (Yokozeki et al., 1990), 6-halo-2′,3′-dideoxypurine nucleosides (Murakami et al., 1991), and other analogs (Hutchinson, 1990). Despite the potential advantages of PNP, nucleoside analogs are still largely synthesized by chemical procedures. The primary difficulties that limit PNP usage include its relatively narrow substrate specificity, its overall stability, and the reversibility of the reaction.

Protein engineering has been used to solve many of the problems that frequently limit the utility of enzymes in the large-scale synthesis of unnatural analogs. For example, disulfide bridges and other modifications have been introduced by mutagenesis and shown to enhance the thermal stability of several enzymes (Pantoliano et al., 1987). A few studies have also successfully used protein engineering to produce large changes in specificity without unduly compromising the catalytic efficiency. Lactate dehydrogenase (Clarke et al., 1989) and xylose isomerase (Meng et al., 1991) represent two examples in which the substrate preference was reversed by protein engineering whereas glutathione reductase (Scrutton et al., 1990) and glyceraldehyde-3-phosphate dehydrogenase (Corbier et al., 1990) represent examples in which engineering led to a shift in coenzyme specificity. In each of these examples, the engineering strategy hinged on the availability of a high-resolution X-ray structure and a detailed knowledge of the catalytic mechanism.

To assess the potential value of X-ray crystallography and site-directed mutagenesis for engineering PNPs with novel substrate specificities, we focused on the design of PNP mutants that efficiently catalyze the synthesis of 6-aminopurine nucleoside analogs, since 6-aminopurine nucleosides are an important class of chemotherapeutic agents and since human PNP has a substrate specificity that favors 6-oxopurines over 6-aminopurines by $>10^4$-fold. Asn243 and Glu201 were targeted as potential sites for mutagenesis based on their involvement in the hydrogen-bonding pattern that inherently favors 6-oxopurines over 6-aminopurines. Since Asn243 is also postulated to play an important role in the catalytic mechanism, amino acid replacements of Asn243 were expected to simultaneously function as a hydrogen bond donor to N7 or risk substantial diminution of catalytic efficiency. Reported herein are human PNP mutants that exhibit remarkable changes in substrate specificity and catalyze the synthesis of 6-aminopurine nucleosides with efficiencies equal to the efficiencies observed for wild-type PNP and 6-oxopurine nucleosides.

MATERIALS AND METHODS

Materials. [^{14}C]-Labeled purine base and nucleoside substrates were purchased from Moravek Biochemicals, Inc., and separated on Cellulose F thin-layer plates from EM Separations. PEG 3350, purines, α-D-ribose 1-phosphate (cyclohexylammonium salt), α-D-2-deoxyribose 1-phosphate (dicyclohexylammonium salt), protease inhibitors, xanthine oxidase (grade III), and calf intestinal adenosine deaminase were obtained from Sigma. Xanthine oxidase was desalted to eliminate 99% of ammonium sulfate by ultrafiltration through Centricon 30 membranes from Amicon. MonoP columns, Polybuffer 74, and the FPLC system were products of Pharmacia.

Preparation of Mutants. Site-directed mutants of human PNP were prepared and overexpressed using the methods described in the previous manuscript (Erion et al., 1997a) and the following oligonucleotides:

Glu201Gln	5′CCC AGC TTT CAG ACT GTG3′
Glu201Ala	5′CCC AGC TTT GCG ACT GTG3′
Asn243Asp	5′CTG ATC ACT GAC AAG GTC AT3′
Asn243Ser	5′CTG ATG ACT AGC AAG GTC ATC3′
Asn243Cys	5′CTG ATC ACT TGC AAG GTC ATC3′
Asn243Gln	5′CTG ATC ACT CAG AAG GTC ATC3′
Asn243Glu	5′CTG ATC ACT GAA AAG GTC ATC3′

Each plasmid was sequenced using the dideoxy methodology of Sanger with the Sequenase kit (U.S. Biochemical) to confirm the mutation. Glu201Ala:Asn243Asp and Glu201Gln:Asn243Asp were constructed by mutation of Glu201 in the Asn243Asp mutant PNP gene.

Expression and Purification of Wild-Type and Mutant hPNP. Wild-type and mutant proteins were overexpressed in *Escherichia coli* strain JM105 or W3110 as described (Erion et al., 1997a). All purification procedures except MonoP chromatography were carried out at 4 °C. The enzymes were purified in two steps. The first fractionation step employed one of the following alternative procedures. (a) Bacteria were suspended in 100 mM Tris, pH 7.4, containing 0.1 M KCl, 2 mM DTT, 0.02% NaN_3, 0.5 mg/mL leupeptin, 2 mg/mL antipain, 2 mg/mL aprotinin, and 0.1 mM PMSF and treated with 1 mg/mL lysozyme. After 40 min, DNase (1 mg/mL) and 5 mM $MgCl_2$ were added and the incubation was continued for an additional 20 min. The suspension was then sonicated for 10 s, supplemented with PMSF to 0.2 mM, and centrifuged 1 h at 120000*g*. The supernatant was fractionated with PEG 3350, and the enzyme was recovered in the 8−20% (w/v) precipitate. (b) Alternatively, some mutants were purified by chromatography on red A agarose as previously described (Erion et al., 1997a). After concentration by ultrafiltration, the proteins were precipitated with 20% PEG 3350. The red A agarose procedure eliminated bacterial PNP in the first step, whereas PEG precipitation did not. Routinely, PNP or mutant PNP that had been purified through the first step was stored at 4 °C overnight or frozen at −80 °C for longer storage. Wild-type PNP was very stable in the form of a partially purified PEG precipitate. Full activity was retained even in PEG precipitates that were held at 37 °C for 7 days.

Enzyme preparations obtained by either of the above procedures were purified further by chromatofocusing, essentially as described previously for erythrocytic PNP (Stoeckler, 1984). The PEG pellet was dissolved in 10 mM HEPES, pH 7, containing 1 mM DTT, and diluted to <5 mg/mL with a 25 mM imidazole-HCl solution (pH 7) containing 0.5 mM DTT. The solution was passed through a 0.22 mm filter and applied to a MonoP HR 5/5 or 5/20 column that was pre-equilibrated and washed with the latter buffer. The enzyme was eluted with Polybuffer 74 (pH 4.5,

0.5 mM DTT). Eluates were monitored for absorbance at 280 nm, and PNP activity was measured as described below. Wild-type and mutant human PNPs all emerged in the expected pH range (6.5−5.5), whereas bacterial PNP was still retained by the column at pH 5.0. The final enzyme preparations obtained from chromatofocusing were concentrated to >1 mg/mL by ultrafiltration. Dilutions were made into a stabilizing buffer of 0.1 M Hepes, pH 7.0, containing 1 mM DTT and either 1 mg/mL bovine serum albumin or 2% PEG 3350. Enzyme concentrations were determined from absorbance at 280 nm, employing $E^{0.1\%} = 0.96$ (Stoeckler et al., 1978) and a subunit mass of 32000 Da.

Steady-State Kinetics. During enzyme purifications, enzymatic activity was detected spectrophotometrically by monitoring either at 293 nm for phosphorolysis using a coupled enzyme assay cocktail containing 0.5 or 5 mM inosine, 50 mM potassium phosphate, and xanthine oxidase (Kalckar, 1947a) or at 258 nm for synthesis of guanosine from 0.1 mM guanine and 2 mM ribose 1-phosphate ($\Delta\epsilon^{mM} = 5.2$; Stoeckler et al., 1980). Synthesis of adenosine and 2'-deoxyadenosine from 76 μM adenine and 1 or 2 mM sugar phosphate was measured spectrophotometrically by a coupled assay employing adenosine deaminase (0.1 unit/mL). Deamination of the reaction products was monitored at 265 nm (Kalckar, 1947b; Agarwal & Parks, 1978). The reaction of 2,6-diaminopurine was monitored directly at 258 nm, $\Delta\epsilon^{mM} = 4.3$. The synthetic reactions were buffered by 100 mM HEPES. Assays were carried out at 30 °C and pH 7.

Unless stated otherwise, kinetic parameters were determined with radioisotope assays. The 50 μL reaction volumes contained 100 mM HEPES, 0.2 mg/mL bovine serum albumin or 2% PEG, various concentrations of a [^{14}C]-labeled purine base or nucleoside substrate (0.013−0.224 μCi), 50 mM potassium phosphate or 10 mM ribose 1-phosphate as second substrate, and sufficient enzyme to permit up to ∼15% conversion to product in the time indicated. For inosine phosphorolysis assays, 0.04 unit/mL xanthine oxidase was added to convert hypoxanthine to uric acid, which gave a better chromatographic separation from inosine. Buffers for determining the pH dependence of adenosine phosphorolysis were morpholinoethane sulfonic acid (pH 5.5, 6, and 6.5) and HEPES (pH 7, 7.5, and 8). Reactions were stopped by addition of 3 μL of 70% perchloric acid and the samples were frozen. Thawed samples were neutralized with 4.5 μL of 5 M K_2CO_3 and microcentrifuged, and 20 μL aliquots were spotted on Cellulose F plates. After elution with 3.8% K_2HPO_4, the percent conversion was quantitated directly by counting on a Berthold Automatic TLC-Linear Analyzer, or spots were excised and counted in Universol ES (ICN Biochemicals, Inc.) with a Packard Tri-Carb 1500 liquid scintillation counter. The latter method was used when percent conversion to product was low. Kinetic parameters were analyzed by nonlinear regression analysis of data fitted to the Michaelis−Menten equation with the Enzfitter computer program (BIOSOFT-Elsevier).

RESULTS

Purification and Characterization of Wild-Type and Mutant PNP Proteins. Plasmids containing the wild-type or mutant human PNP genes were constructed as described, and the proteins were overexpressed in *E. coli*. Purification was achieved in two steps. Two alternative purification procedures, red A agarose chromatography followed by chromatofocusing (Erion et al., 1997a) and PEG fractionation followed by chromatofocusing (described herein) yielded enzyme preparations of similar specific activity. Chromatofocusing profiles of enzymes purified initially by PEG fractionation showed more UV-absorbing peaks. However, virtually the same final purification was achieved. For example, a batch of Glu201Ala prepared by PEG fractionation differed in specific activity by only 5% from a batch prepared using the red A agarose procedure. Electrophoresis of the mutant proteins on native and denaturing polyacrylamide gels indicated retention of quaternary structural integrity and migration consistent with the expected electrostatic effects of the substitutions. In general, wild-type and mutant enzyme preparations showed good stability, although occasionally the concentrated stock solutions lost 20−50% activity within 24 h of standing at 4 °C or after a freeze−thaw cycle despite the presence of DTT. The reaction velocities of the wild-type and mutant enzymes with radiolabeled purine bases and ribonucleosides were determined using a saturating or near-saturating concentration of the second substrate, and reaction time courses in which substrate conversion was limited to <15%. The steady-state kinetic parameters are presented in Tables 1, 2, and 3.

Kinetics of Wild-Type PNP. The recombinant enzyme has similar affinities for the preferred substrates, inosine, hypoxanthine, guanosine, and guanine (Table 1). The catalytic rates per active site (k_{cat}) are also similar (Table 2) with yield efficiencies (k_{cat}/K_M) of $>10^6$ s^{-1} M^{-1}. The catalytic efficiency for adenine was 10000-fold lower than for hypoxanthine, and the catalytic efficiency for adenosine was 350,000-fold lower than for inosine (Table 3).

Kinetics of Glu201 Mutants. To assess the contribution of the Glu201 carboxylate group to substrate discrimination, the kinetics of the Glu201Ala mutant was determined for a variety of substrates. As shown in Tables 1 and 2, the Glu201Ala mutant exhibited very large detrimental changes in K_M and k_{cat} for the normal substrates. The effects of this mutation were most pronounced with guanosine and guanine. Guanosine efficiency decreased by 6 orders of magnitude (Table 3). Similar decreases in efficiency were found for guanine; although, the true kinetic parameters were not determined because of limiting solubility. No reaction was detected with 1.3 mM adenosine (despite the use of 22-fold more enzyme and a 40-fold longer incubation time than employed with inosine). The affinity for adenine was not altered significantly, whereas the preference for hypoxanthine over adenine was reduced by a factor of >50. Relative to the wild-type rate, the reaction velocity was reduced to 1.7%.

Mutation of Glu201 to Gln produced K_M values in the 10^{-2}−10^{-3} M range for all 6-oxopurine substrates except guanine, which exhibited an exceptionally low K_M value of 79 μM (Table 1). The k_{cat} values for all substrates were reduced to 0.6−5% of those obtained with the wild-type enzyme. In general, the Glu201Gln mutant was a better catalyst than the Glu201Ala protein, especially with guanosine and guanine. Both mutants were 20−200-fold more efficient in nucleoside synthesis than phosphorolysis, which is substantially greater than the 4−6-fold preference displayed by the wild-type enzyme. The Glu201Gln mutant displayed reduced efficiencies with 6-aminopurines, relative to wild-type PNP, and retained selectivity for 6-oxopurine substrates. Phosphorolysis of 600 μM adenosine was

Table 1: K_M (μM) Values for Wild-Type and Mutant PNPs[a]

enzyme	Ino	Hyp	Guo	Gua	Ado	Ade
wild-type	45 ± 7	10 ± 1	12 ± 1	6 ± 0.7	650 ± 79	440 ± 11
Glu201Ala	10 000 ± 3000	450 ± 35	1400 ± 430	nd[b]	nd[c]	320 ± 31
Glu201Gln	>10000	1400 ± 50	3300 ± 5	79 ± 13	nd[d]	1100 ± 50
Asn243Ala	21 ± 3	480 ± 1	42 ± 0.1	120 ± 17	nd[d]	170 ± 10
Asn243Asp	340 ± 33	60 ± 11	140 ± 20	50 ± 5	740 ± 60	71 ± 5
Glu201Ala:Asn243Asp	1800 ± 450	1200 ± 260	200 ± 20	410 ± 39	1100 ± 90	93 ± 4
Glu201Gln:Asn243Asp	11 000 ± 100	1250 ± 150	500 ± 200	160 ± 60	930 ± 2	65 ± 5

[a] Kinetic parameters for wild-type and mutant enzymes were determined under comparable conditions except that enzyme concentration and reaction times were adjusted. nd = not determined. [b] K_M exceeded solubility. [c] No activity detected. [d] Activity detected; efficiency below that of wild-type PNP.

Table 2: k_{cat} (s^{-1}) Values for Wild-Type and Mutant PNPs[a]

enzyme	Ino	Hyp	Guo	Gua	Ado	Ade
wild-type	57 ± 5	70 ± 3	28 ± 2	48 ± 1	0.0024 ± 0.0002	0.31 ± 0.01
Glu201Ala	0.50 ± 0.01	1.5 ± 0.4	0.003 ± 0.0005	nd[b]	nd[c]	0.0051 ± 0.0001
Glu201Gln	>1	3.8 ± 0.1	0.17 ± 0.01	0.8 ± 0.06	nd[d]	0.0069 ± 0.0003
Asn243Ala	0.054 ± 0.002	16.4 ± 0.1	0.051 ± 0.001	26 ± 1	nd[d]	0.0029 ± 0.0004
Asn243Asp	2.3 ± 0.1	14 ± 3	2.8 ± 0.4	29 ± 1	12 ± 3	36 ± 5
Glu201Ala:Asn243Asp	0.000 78 ± 0.000 01	0.030 ± 0.001	0.000 09 ± 0.000 05	0.003 ± 0.002	0.47 ± 0.02	26 ± 1
Glu201Gln:Asn243Asp	0.017 ± 0.001	0.057 ± 0.003	0.0006 ± 0.0001	0.009 ± 0.0007	0.67 ± 0.03	7.2 ± 0.2

[a] Kinetic parameters for wild-type and mutant enzymes were determined under comparable conditions except that enzyme concentration and reaction times were adjusted. nd = not determined. [b] K_M exceeded solubility. [c] No activity detected. [d] Activity detected; efficiency below that of wild-type PNP.

Table 3: Catalytic Efficiencies, k_{cat}/K_M (s^{-1} M^{-1}) for Wild-Type and Mutant PNPs[a]

enzyme	Ino	Hyp	Guo	Gua	Ado	Ade
wild-type	1.3×10^6	7.0×10^6	2.3×10^6	7.5×10^6	3.7	7.0×10^2
Glu201Ala	5×10^1	3.3×10^3	2.4	nd[b]	nd[c]	1.6×10^1
Glu201Gln	$\sim 1 \times 10^2$	2.7×10^3	5.2×10^1	1.0×10^4	nd[d]	6.1
Asn243Ala	2.6×10^3	3.4×10^4	1.2×10^3	2.1×10^5	nd[d]	1.7×10^1
Asn243Asp	6.7×10^3	2.4×10^5	2.0×10^4	5.8×10^5	1.6×10^4	5.1×10^5
Glu201Ala:Asn243Asp	4.5×10^{-1}	2.4×10^1	4×10^{-1}	6.6	4.3×10^2	2.8×10^5
Glu201Gln:Asn243Asp	1.5	4.6×10^1	1	5.4×10^1	7.2×10^2	1.1×10^5

[a] Kinetic parameters for wild-type and mutant enzymes were determined under comparable conditions except that enzyme concentration and reaction times were adjusted. nd = not determined. [b] K_M exceeded solubility. [c] No activity detected. [d] Activity detected; efficiency below that of wild-type PNP.

detected only with enzyme concentrations 16-fold higher than those used to study adenosine with wild-type PNP, and kinetic parameters were not determined. Substrate affinity for adenine decreased 2.5-fold, while k_{cat} dropped to ~2% of the wild-type value (Tables 1 and 2). Since efficiency was reduced less profoundly with adenine than with hypoxanthine, the preference for hypoxanthine is decreased by a factor of ~20 relative to wild-type PNP.

Kinetics of Asn243 Mutants. Mutants were prepared to assess the role of the Asn243 carboxamido group in purine base specificity. Mutation of Asn243 to Ala resulted in decreased k_{cat} values relative to wild-type for both inosine and guanosine (0.2%), but had very modest effects on the substrate affinities (Tables 1 and 2). In the synthesis direction, the k_{cat} values with hypoxanthine and guanine decreased slightly (46−77%), whereas overall catalytic efficiency decreased by ~200- and 36-fold, respectively, due to 48- and 19-fold increases in K_M. In contrast, the Asn243Ala mutant exhibited a modest 2.6-fold improvement in substrate affinity for adenine. This modest gain in substrate binding, however, was offset by a large drop in the adenine k_{cat} value (1% of wild-type). The overall ~40-fold decrease in catalytic efficiency exhibited by the Asn243Ala mutant for adenine was therefore less than the ~200-fold decrease in efficiency exhibited by the Asn243Ala mutant for hypoxanthine. Accordingly, the substrate preference for hypoxanthine over adenine by the Asn243Ala mutant was about 5-fold less than that exhibited by wild-type PNP (Table 3). The Asn243Ala mutation was also detrimental to adenosine phosphorolysis with the catalytic efficiency even lower than for wild-type PNP, and consequently, the kinetic parameters were not determined.

Mutation of Asn243 to Asp resulted in a dramatic reversal of substrate specificity (Tables 1−3). Relative to wild-type PNP, the Asn243Asp mutant exhibited 200- and 100-fold lower efficiencies with inosine and guanosine, respectively, due to adverse effects on both K_M and k_{cat}. Similarly, respective 29- and 13-fold losses in efficiency were found with hypoxanthine and guanine due to ~10-fold decreases in affinity and lesser changes in k_{cat}. In contrast, the catalytic rate with adenosine increased 5000-fold resulting in a 2-fold preference for adenosine relative to inosine for the Asn243Asp mutant. Similarly a 6-fold lower K_M and a 116-fold improvement in k_{cat} for adenine raised the Asn243Asp catalytic efficiency to 5×10^5 s^{-1} M^{-1}, which is a value 2-fold higher than that for hypoxanthine with this enzyme. These results represent an 800000-fold shift in substrate preference in that the wild-type enzyme favors inosine over adenosine by 350000-fold and the Asn243Asp mutant favors adenosine over inosine by 2.4-fold.

The mutant protein also catalyzed the synthesis of 2′-deoxyadenosine with high efficiency. Spectrophotometric

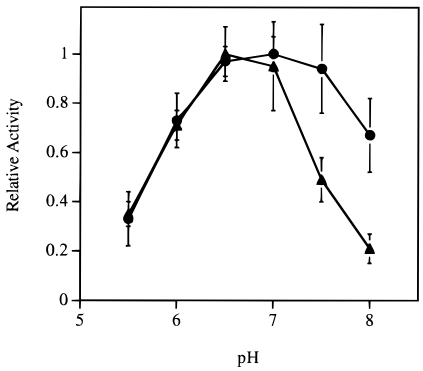

FIGURE 1: The pH dependence of adenosine phosphorolysis with wild-type and Asn243Asp PNP. Percent conversion to adenine was determined as described in Materials and Methods. Results for wild-type (●) and Asn243Asp (▲) PNP are expressed as a fraction of the maximal activity obtained with that enzyme and are the mean ± SD of four determinations.

assays with 76 μM adenine ($\sim K_M$ concentration) indicated that reaction with 1 mM 2-deoxyribose 1-phosphate is 1.3-fold faster than with 2 mM ribose 1-phosphate (data not shown). A similar ratio was obtained with guanine as substrate.

In addition to Asn243Ala and Asn243Asp, the following seven mutants were prepared and screened for activity at pH 7 with 1 mM nucleoside (inosine or adenosine) and 50 mM phosphate: Asn243Gln, Asn243Glu, Asn243Ser, Asn243Cys, Asn243His, Asn243Gly, and Asn243Leu. None of the mutants were found to exhibit catalytic activity greater than Asn243Ala.

pH Dependence of Adenosine Phosphorolysis. The Asn243Asp mutant retained 20−60% of wild-type catalytic activity with 6-oxopurine bases and 4−10% with their nucleosides, while k_{cat} with adenosine was improved 5000-fold. To assess the dependence of catalytic rate on the ionization state of the Asp, the pH dependence of adenosine phosphorolysis was determined for wild-type PNP and the Asn243Asp mutant. An adenosine concentration (600 μM) near the K_M values of both enzymes was used to make the assay highly sensitive to changes in both K_M and V_{max}. As Figure 1 indicates, the wild-type enzyme retained high activity at pH 7.5, whereas the mutant exhibited a steep decline in relative activity above pH 7.0. Both enzymes exhibited a similar rate of decline in activity at pHs below pH 6.5.

Kinetics of Glu201::Asn243 Double Mutants. Two double mutants were prepared, namely Glu201Ala::Asn243Asp and Glu201Gln::Asn243Asp, and characterized using a variety of substrates (Tables 1−3). Relative to wild-type PNP, the Glu201Ala::Asn243Asp double mutant displayed approximately 10^6- and 10^5-fold lower efficiencies with 6-oxopurine nucleosides and bases, respectively. The resultant 10^4-fold lower efficiencies with these substrates relative to the Asn243Asp single mutant indicated additive detrimental effects of the double mutation. Concomitantly, the Glu201Ala ::Asn243Asp double mutant displayed a catalytic efficiency for adenine nearly equal to the Asn243Asp single mutant while the catalytic efficiency for adenosine was 37-fold lower. The net result was a 1000−10000-fold preference for 6-aminopurines (Table 3) and a 10^8-fold change in substrate preference relative to wild-type PNP. The kinetic constants obtained with Glu201Gln:Asn243Asp were similar to those obtained with Glu201Ala:Asn243Asp.

Table 4: Kinetic Parameters for 2,6-Diaminopurine with Wild-Type and Mutant PNPs[a]

enzyme	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M (s^{-1} M^{-1})
wild-type	nd[b]	nd[b]	nd
Asn243Asp	45 ± 8	65 ± 8	1.4×10^6
Glu201Ala:Asn243Asp	240 ± 90	5.4 ± 1.4	2.3×10^4
Glu201Gln:Asn243Asp	180 ± 44	12 ± 2	6.5×10^4

[a] Kinetic parameters were determined spectrophotometrically, as described in Materials and Methods. nd = not determined. [b] No activity detected.

Kinetics of 2,6-Diaminopurine. The 6-aminopurine substrate, 2,6-diaminopurine, was studied with the Asn243Asp mutants since it is a 6-aminopurine analog of guanine and therefore combines the hydrogen bond patterns of adenine and guanine. As shown in Table 4, no detectable activity was observed for wild-type PNP and 2,6-diaminopurine whereas the Asn243Asp mutant utilized 2,6-diaminopurine as a substrate with an efficiency (1.4×10^6 s^{-1} M^{-1}) ~3-fold higher than adenine and on the same order of magnitude as the efficiencies calculated for hypoxanthine or guanine and wild-type PNP. The results in Table 4 also show that 2,6-diaminopurine has approximately the same affinity for both the Glu201Gln::Asn243Asp and Glu201Ala::Asn243Asp double mutants. The efficiency of 2,6-diaminopurine with the double mutants, however, was 20−60-fold lower than with Asn243Asp. Nevertheless, the double mutants displayed an 1000−3500-fold preference for 2,6-diaminopurine relative to hypoxanthine and guanine (Tables 3 and 4), respectively.

DISCUSSION

Rationale for Mutant Design. Human PNP exhibits a high preference for 6-oxopurine nucleosides relative to 6-aminopurine nucleosides. Consistent with these findings, recombinant wild-type PNP exhibited a 350000-fold preference for inosine over adenosine (Table 3). The decreased preference for adenosine was attributed primarily to a k_{cat} value that was <0.005% of the value obtained for inosine (Table 2). In the synthesis direction, PNP catalyzed the synthesis of adenosine 10000-fold less efficiently than inosine. In this case, the lower efficiency reflected a decrease in both k_{cat} (<0.5%) and a >40-fold increase in K_M.

Efforts to engineer a PNP with a substrate specificity that favors 6-aminopurine nucleosides required the identification of residues that reversed the natural N1−O6 hydrogen bond donor−acceptor pattern while maintaining catalytic activity. Accordingly, a hydrogen bond acceptor was required at position 243 whereas a hydrogen bond donor was required at position 201. Since Glu201 and Asn243 are important for catalysis as well as for substrate recognition, residues replacing them were also required to provide the essential functionality necessary for catalysis. Thus, if the mechanism postulated in the preceding paper is correct (Erion et al., 1997b), residues that replace Asn243 would have to donate a hydrogen bond to N7 in the TS in order to obtain optimal catalytic activity.

Glu201Ala and Asn243Ala Mutants. The dependence of PNP on Asn243 and Glu201 for efficient catalysis of 6-oxopurine substrates was established previously (Erion et al., 1997a). To evaluate the role of these residues in PNP's high substrate preference for 6-oxopurines over 6-aminopu-

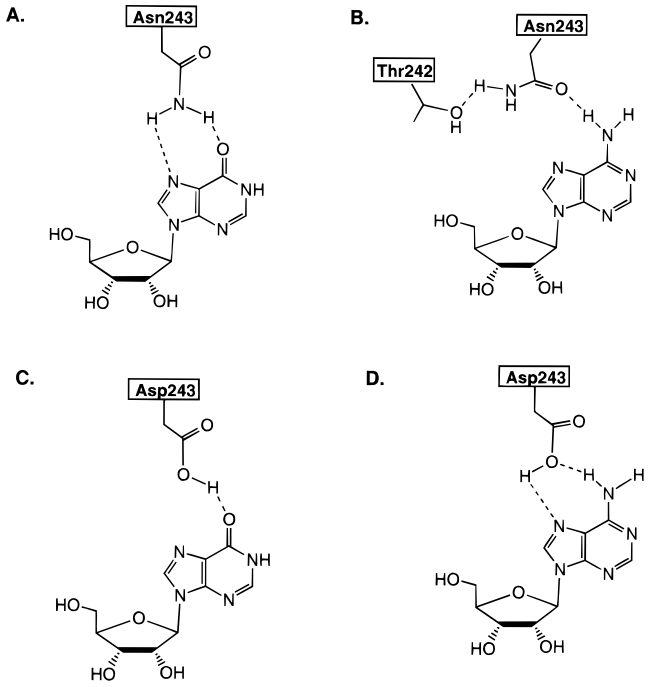

FIGURE 2: Hydrogen bond interactions that confer purine base specificity in wild-type PNP and the Asn243Asp mutant. Upper left panel shows inosine in the active site of wild-type PNP; upper right panel displays a possible alteration in the hydrogen bond pattern for wild-type PNP in the presence of adenosine; lower left panel shows the hypothetical orientation of inosine in the active site of the Asn243Asp mutant; lower right panel shows the hypothetical orientation of adenosine in the active site of the Asn243Asp mutant.

rines, kinetic parameters were determined for adenosine synthesis and adenosine phosphorolysis using the corresponding alanine mutants. Similar to earlier results, the catalytic efficiency of the Glu201Ala mutant for 6-oxopurine nucleosides (inosine and guanosine) decreased by 5 orders of magnitude while the efficiencies of the Asn243Ala mutant for 6-oxopurine nucleosides decreased by 3 orders of magnitude (Table 3). Adenosine phosphorolysis was also decreased with catalytic efficiencies even less than the very low values exhibited for the wild-type enzyme. In the synthesis direction, catalytic efficiencies for both mutants with adenine were approximately 40-fold lower than for wild-type and 200−2000-fold lower than the efficiencies determined for wild-type and hypoxanthine. Although replacement of these residues with alanine appeared to slightly enhance specificity for 6-aminopurines, the large loss in k_{cat} resulted in enzymes with poor efficiencies.

Asn243Asp Mutant. Further efforts to design a PNP mutant with specificity for 6-aminopurine nucleosides led to the selection of Asp as a suitable replacement for Asn. On the basis of predicted hydrogen bond patterns (Figure 2), the Asn243Asp mutant was expected to favor 6-aminopurine over 6-oxopurine nucleosides. As indicated by Figure 2D, a protonated Asp243 could accept a hydrogen bond from the 6-amino group and simultaneously donate a hydrogen bond to N7 as required for transition state stabilization. The expected pK_a of a buried Asp (Haschenmeyer & Haschenmeyer, 1974) suggests that the carboxylate group can exist in the protonated form at pH 7 to a significant extent. In contrast, 6-oxopurines are unable to form two simultaneous hydrogen bonds between O6, N7, and the Asp residue. Since the O6 carbonyl is a better hydrogen bond acceptor than N7, the Asp carboxylate is likely to favor hydrogen bond formation with O6 (Figure 2C). Consequently, decreased catalytic activity was predicted for 6-oxopurine nucleosides, since the preferred orientation of Asp would not result in a hydrogen bond to N7 in the TS. Kinetic analysis of the Asn243Asp mutant with both inosine and guanosine supported the predicted hydrogen bond mismatch. As shown in Tables 1−3, the Asn243Asp mutant exhibited a larger K_M and a smaller k_{cat} for both inosine and guanosine relative to wild-type with the combined effect resulting in a decrease in catalytic efficiency of 2−3 orders of magnitude.

In contrast to results with inosine and guanosine, adenosine was an excellent substrate for the Asn243Asp mutant. The major difference between the Asn243Asp mutant and wild-type PNP was the 5000-fold increase in k_{cat} (Table 2). Accordingly, the catalytic efficiency of the Asn243Asp mutant with adenosine is ∼4000-fold better than that of wild-type PNP and only 80-fold lower than the efficiency of wild-type PNP with inosine. Furthermore, the substrate specificity for the Asn243Asp mutant is the reverse of wild-type PNP with the Asn243Asp mutant favoring adenosine over inosine by 2.4-fold and the wild-type PNP favoring inosine over adenosine by 350000-fold. Consequently, the change in overall preference for adenosine is ∼800000. These results are consistent with the hydrogen bond patterns shown in Figure 2.

The importance of Asn and Asp in catalysis is further supported by several experimental findings. First, replacement of Asn243 with other amino acids (e.g., Ala, Ser, Cys, His, Gly, Gln, and Glu) resulted in very low activity for both 6-oxopurine and 6-aminopurine nucleoside substrates. Second, analysis of amino acid sequences for PNPs with specificity for 6-oxopurines showed strict conservation of Asn243 (Erion et al., 1997b) whereas a sequence for a 6-aminopurine specific nucleoside phosphorylase, methylthioadenosine phosphorylase, had an Asp at the same position as Asn243 (Takabayashi, K., unpublished result). Last, the presence of a protonated Asp is supported by analysis of the pH dependence of adenosine phosphorolysis. As indicated in Figure 1 and in contrast to wild-type PNP, the catalytic rate of the Asn243Asp mutant rapidly diminishes above pH 7. Although this loss in activity could arise from either a decrease in binding affinity, a decrease in catalytic rate, or both, it is at least consistent with the Asn243Asp mutant having a new titratable acidic residue with catalytic importance.

Glu201Gln Mutant. Hydrogen bond donation by N1 of 6-oxopurine nucleosides to Glu201 suggested that replacement of Glu201 with Gln could reverse the pattern by providing a hydrogen bond donor compatible with N1 of 6-aminopurine nucleosides. As expected, the Glu201Gln mutation was detrimental to inosine and guanosine phosphorolysis. On the basis of these results, rotation of the Gln side-chain carboxamide in a manner that would position the carbonyl near the N1 hydrogen of inosine was apparently not possible or of no significant benefit to catalysis. Surprisingly, the Glu201Gln mutant also exhibited a similar detrimental effect on both K_M and k_{cat} with adenosine. In fact, adenosine is a better substrate for the wild-type enzyme than for the Glu201Gln mutant (Table 3). Possible explanations are that a protonated Glu201 is required for catalysis or that Gln is unable to form optimum hydrogen bonds with

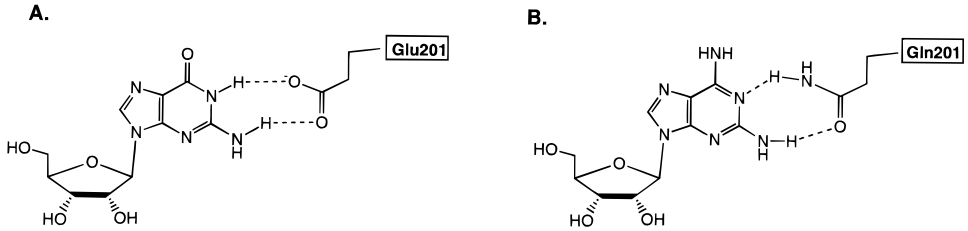

FIGURE 3: Hydrogen bond pattern predicted for wild-type PNP and inosine (A) and for the Glu201Gln mutant and adenosine (B).

adenosine. The former explanation is inconsistent with the proposed catalytic mechanism (Erion, et al., 1997b). The latter explanation could be the case if the Gln side chain failed to maintain the same orientation as Glu. This could result from the inherent difference in charge and hydrophobicity between the Glu and Gln side chains. For example, the Gln carboxamido group can donate two hydrogen bonds, which will only be partially fulfilled if the Gln side chain is positioned identically to Glu. Similarly, the decrease in anionic charge on the side chain may disfavor the same orientation as the Glu side chain due to an electrostatic incompatibility of the neutral carboxamido group in a region of the protein that normally accommodates a negatively charged carboxylate. Alternatively, the Gln side chain may be positioned identically to Glu but result in a significant decrease in catalytic efficiency due to a difference in hydrogen bond strength. Numerous studies indicate that a hydrogen bond involving a charged residue [e.g., Glu-COO^-·(H)N1] is considerably stronger than a hydrogen bond between two neutral species (e.g., Gln-$CONH_2$·N1) (Fersht, 1985).

Double Mutants. To further enhance substrate preference of the Asn243Asp mutant for 6-aminopurines, we prepared double mutants in which Glu201 was changed to either Ala or Gln. For the Glu201Ala::Asn243Asp mutant, we expected that the beneficial effects of Asp243 would be retained while the replacement of Glu201 with Ala would weaken binding to 6-oxopurines and possibly aid binding of 6-aminopurines due to removal of the negatively charged hydrogen bond acceptor near N1. The Glu201Gln::Asn243Asp mutant was expected to enhance the preference for 6-aminopurines based on formation of a potential hydrogen bond between the carboxamido group and N1. In contrast to expectations, both double mutants showed a reduction in efficiency for adenosine by 2 orders of magnitude relative to the Asn243Asp mutant. Both double mutants, however, were still 100-fold more efficient for adenosine phosphorolysis than wild-type PNP and exhibited $>10^8$-fold enhancement in substrate preference for adenosine over inosine relative to wild-type PNP. The poor catalytic efficiency of the double mutants again demonstrated the importance of the Glu201−substrate interaction in catalysis.

Synthesis of 2,6-Diaminopurine Riboside. X-ray structures of PNP−guanine complexes indicated that the carboxyl group of Glu201 forms two hydrogen bonds: one to N1 and the other to the 2-amino group via the second carboxyl oxygen. A similar hydrogen bond pattern exists in *ras* p21, which uses an Asp to form a hydrogen bond with N1 and the 2-amino group of GDP (Wolley & Clark, 1989; Valencia et al., 1991). The hydrogen bond to the 2-amino group of GDP appears to contribute significantly to substrate binding in *ras* based on the >50-fold discrimination of GDP over IDP (Sigal et al., 1986). In contrast, PNP exhibits a much more modest 4-fold preference for guanosine over inosine. Nevertheless, 2,6-diaminopurine was studied as a possible substrate for the double mutant since the 2-amino group could strengthen the interaction between Gln and 6-aminopurines and thereby enhance catalysis by orienting the Gln carboxamide in a manner that favors formation of a hydrogen bond to N1 (Figure 3B). As shown in Table 4, the efficiency of 2,6-diaminopurine with Glu201Gln::Asn243Asp was only 2-fold higher than with Glu201Ala::Asn243Asp. Furthermore, both double mutants utilized 2,6-diaminopurine ~10-fold less efficiently than adenine. Interestingly, 2,6-diaminopurine was a very good substrate for the Asn243Asp mutant, which exhibited a catalytic efficiency (1.4×10^6 s^{-1} M^{-1}) on the same order of magnitude as wild-type PNP with its natural substrates hypoxanthine and guanine. These results suggest that the Gln side chain fails to form a strong hydrogen bond to N1 and thereby contribute to substrate binding and catalysis. It further suggests that alternative orientations exist for the Glu201 side chain in the Asn243Asp mutant which minimize the detrimental contact between N1 and the carboxylic anion and enable productive binding of adenine and adenine analogs.

Amino Acid Sequence Analysis. The importance of Glu201 and Asn243 in human PNP substrate recognition and catalysis is further supported by their strict conservation in all other PNPs that exhibit specificity for 6-oxopurine nucleosides (Erion et al., 1997b). Evidence that Asp is an amino acid substitution that can aid recognition and catalysis of 6-aminopurine nucleosides is suggested by the sequence of the recently cloned (Takabayashi, K., unpublished data) human 5′-methylthioadenosine (MTA) phosphorylase gene (Olopade et al., 1995). A comparison of the amino acid sequences for MTA phosphorylase and PNP revealed significant homology and thereby a high probability that the proteins will have some similarity in their overall structure and active-site architecture. One striking difference between the sequences was the presence of an Asp and a Ser in the MTA phosphorylase at the positions in the PNP sequence corresponding to Asn243 and Glu201, respectively. On the basis of results for the Asn243Asp mutant, the Asp in MTA phosphorylase may account for the high substrate specificity of MTA phosphorylase for 6-aminopurines and prove to be essential for efficient catalysis.

Conclusions. Crystallographic and site-directed mutagenesis studies on PNP identified Glu201 and Asn243 as active-site residues essential for catalysis and responsible for the high preference of human PNP for 6-oxopurines. Results reported in this manuscript provide further support for the proposed catalytic mechanism (Erion et al., 1997b) and for the origin of the 6-oxopurine preference. Replacement of Asn with Asp produced a mutant PNP with a 4300-fold higher catalytic efficiency for adenosine relative to wild-type and an 800000-fold change in preference for adenosine

over inosine relative to wild-type. The double mutant, Asn243Asp::Glu201Gln, exhibited a 190-fold increase in catalytic efficiency with adenosine relative to wild-type PNP, a 480-fold preference for adenosine relative to inosine, and a 1.7×10^8-fold change in preference for adenosine over inosine relative to wild-type PNP. The Asn243Asp mutant was also shown to synthesize 2,6-diaminopurine riboside with a catalytic efficiency (1.4×10^6 s^{-1} M^{-1}) on the same order of magnitude as wild-type PNP with its natural substrates hypoxanthine and guanine. Unlike many previous protein engineering studies, these results demonstrate that alterations in the substrate binding site by site-directed mutagenesis can reverse substrate specificity while maintaining high catalytic efficiency. The reversal in substrate specificity from 6-oxopurine analogs to 6-aminopurine analogs was particularly remarkable since it entailed replacement of a residue involved in TS stabilization (i.e., Asn243). The successful engineering of PNP for 6-aminopurine specificity suggests that PNP can be tailored to accommodate a diverse set of substrates and thereby be of potential value as a catalyst for the synthesis of purine nucleoside analogs.

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