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Effect of the phosphate substrate on drug-inhibitor binding to human purine nucleoside phosphorylase

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ABSTRACT

The thermodynamics of the drug-inhibitors acyclovir, ganciclovir, and 9-benzylguanine binding to human purine nucleoside phosphorylase (hsPNP) were determined from isothermal titration calorimetry as a function of the substrate phosphate ion (Pi) concentration from 0 to 0.125 M and temperature from 15 °C to 35 °C. At 25 °C and with an increase in the Pi concentration from 0 to 50 mM, acyclovir binding becomes more entropically-driven and ganciclovir binding becomes more enthalpically-driven. At 25 °C, the tighter 9-benzylguanine binding reaction goes from an enthalpically-driven reaction in the absence of Pi to an entropically-driven reaction at 10 mM Pi, and the enthalpically-driven nature of the binding reaction is restored at 75 mM Pi. Since the dependencies of the driving-nature of the binding reactions on Pi concentration can be simulated by Pi binding to its catalytic site, it is believed that bound Pi affects the interactions of the side-chains with the ribose catalytic site. However, the binding constants are unaffected by change in the bound Pi concentration because of enthalpy–entropy compensation. The enzymatic activity of hsPNP was determined by an ITC-based assay employing 7-methylguanosine and Pi as the substrates. The heat of reaction determined from the assay increased by 7.5 kJ mol^{-1} with increase in Pi concentration from 50 to 100 mM and is attributed to weak binding of the Pi to a secondary regulatory site. Although the binding constants of acyclovir and ganciclovir at 20 μM hsPNP were in agreement with the inverse inhibition constants determined from the ITC enzyme inhibition assays at 60 nM, the binding constant of 9-benzylguanine, which interacts with Phe159 from an adjacent subunit, decreased from 5.62×10^5 M^{-1} to 1.14×10^5 M^{-1}. This reduction in the 9-benzylguanine binding affinity along with a 7-fold increase in the specific activity of hsPNP at 14.5 nM results from partial dissociation of the hsPNP trimer into monomers below the 60 nM level.

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Introduction

Many drug-targeted enzymes, particularly the regulatory enzymes, are multi-substrate enzymes where it is not known how the potency of a drug-inhibitor targeting one substrate site is affected by the presence of the other substrate in an adjacent site. Many bi-substrate enzymes exhibit "substrate activation phenomenon" where the enzymatic reaction of the enzyme at low concentrations of one substrate is accelerated in the presence of the second substrate. Purine nucleoside phosphorylase (PNP)[1] is an enzyme that utilizes ribo- and deoxyribonucleosides and phosphate in adjacent sites to catalyze the reversible cleavage of the *N*-glycosidic bond of the ribo- and deoxyribonucleosides and subsequent phosphorylation of the ribose moiety as shown [1,2] here in Fig. 1. PNP exhibits substrate activation phenomenon where the slow hydrolysis of the β-purine nucleoside is accelerated by the presence of phosphate ions (Pi) in the buffer solution. A decrease in the inhibition potency by drug-inhibitors is also observed for human PNP (hsPNP) in the presence of high concentrations Pi [1].

The enzyme reaction catalyzed by PNP is important in the salvage pathway for recycling purine ribo-and deoxyribonucleosides to generate free purine bases and is an alternative to the de novo purine biosynthetic pathway [3]. The enzymatic function of human PNP (hsPNP) has important medical implications. It was first described by Giblett et al. in 1975 as a rare autosomal recessive cellular immunodeficiency disorder in that hsPNP deficiency in humans leads to an impairment of T-cell function, usually with no apparent effects on B-cell function [1]. Although the mechanism by which hsPNP deficiency causes impaired T-cell maturation and differentiation is not completely understood, T-cell leukemias and lymphomas can be impaired by designing efficient inhibitors that target hsPNP. This has lead to drug inhibition targeting of hsPNP for chemotherapeutic intervention in the treatment of cancer [2]. In addition, targeted anticancer gene therapy is being developed

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[1] *Abbreviation used:* hsPNP, human purine nucleoside phosphorylase; Pi, phosphate ion; PNP, purine nucleoside phosphorylase, bsPNP, calf's spleen PNP; ITC, isothermal titration calorimetry; BSA, bovine serum albumin.

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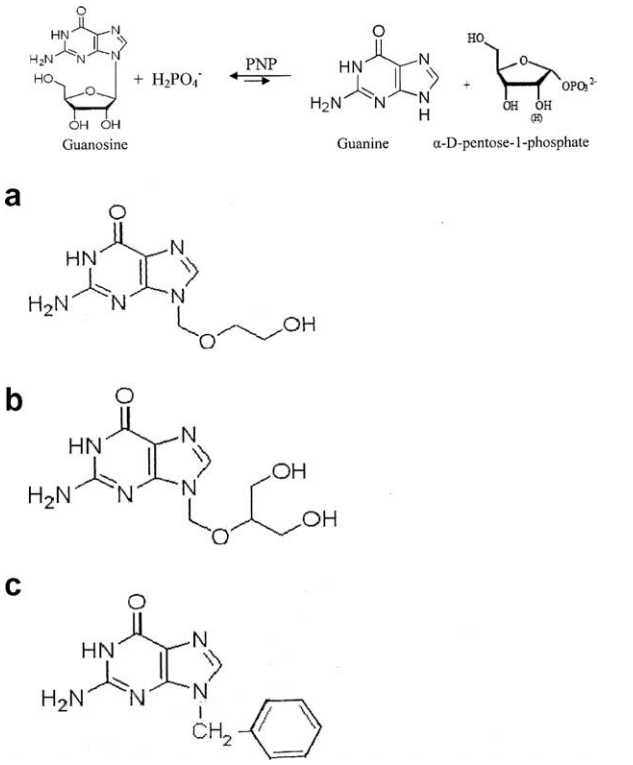

Fig. 1. A schematic representation of the reaction catalyzed by purine nucleoside phosphorylase and structures of the drug-inhibitors for hsPNP (a) acyclovir; (b) ganciclovir; (c) 9-benzylguanine.

by exploiting the distinct substrate specificity between *Escherichia coli* PNP and hsPNP where adenosine is exclusively a natural substrate of the *E. coli* PNP [2]. The strategy here is to transfect human tumors with the gene for *E. coli* PNP to express the *E. coli* PNP in the tumor where it exclusively recognizes a chemotherapeutic prodrug of adenosine and subsequently cleaves the prodrug to an active, cytotoxic form that kills the tumor cells [2,4]. HsPNP has also been considered an attractive drug target in the treatment of other T-cell related autoimmune diseases, e.g. rheumatoid arthritis, and multiple sclerosis [2]. Furthermore, the role of hsPNP in T-cell mediated immunity makes it a drug target for the suppression of the host-graft response in organ transplantation [2]. The X-ray crystal structures of PNP from different organisms including various bacterial species show that *E. coli* PNP and S*almonella typhimurium* PNP are hexameric whereas mammal PNP is trimeric [1].

The crystal structure for hsPNP [5] as well as its structures with bound drug-inhibitors exhibit the following characteristics: the enzyme is an active trimer with three independent substrate binding sites located near the subunit interface: the purine-binding site, composed of Ala116, Phe200, Glu201, Val217, Met219, Thr242, and Asn243, is proximal to the ribose-binding site, composed of Tyr88, Phe159, Phe200, His86, His257, and Met219, and to the catalytic Pi-binding site, composed of Ser33, Arg84, His86, and Ser220. The residues Phe200 and Met219 are involved in binding of the purine and ribose, while His86 is involved in binding of both the ribose and Pi. There is evidence from the X-ray crystallography of hsPNP for an additional Pi-binding site, possibly a Pi regulatory-site that is composed of Gln144 and Arg148 [5]. The hsPNP binding sites are near the subunit interface so that residues Phe159 and Arg148 are from the adjacent subunit [6,7]. A loop composed of residues 241–260 in the hsPNP crystal structure acts as a gate that opens upon substrate or drug-inhibitor binding [7–10]. This loop, which includes residues Thr242 and Asn243 from the purine-binding site and His257 from the ribose-binding site, becomes highly ordered upon substrate binding [11]. The mutation of Tyr249 of the loop to tryptophan in hsPNP yielded a maximum Trp fluorescence peak red-shifted to 358 nm, indicating that the loop is exposed to the solvent [11].

The role of the second substrate, Pi, is more complex since it also facilitates the rate-limiting release of the tightly-bound purine bases in PNP. [11,12] However, Pi also exhibits negative cooperativity in the calf spleen PNP (bsPNP) enzyme reaction, which was earlier attributed toward a conformational change, promoting dissociation to a more active monomer, or coupling between two Pi sites on the same monomer subunit [7]. A decrease of the Pi concentration from 50 mM to 1 mM, increased the binding affinity of the multiphosphorylated acyclovir drug-inhibitors to hsPNP [13]. The fluorescence intensity of Trp249 of the Tyr249→Trp mutant of hsPNP increased upon saturation with bound Pi, indicating that Pi causes only a change in the environment of Tyr249 while retaining the water solvent exposure of the loop [11]. Previously performed enzyme kinetic studies on calf's spleen PNP (bsPNP) also reported an increase in specific bsPNP activity upon dissociation of the enzyme and the dissociation was enhanced at higher Pi concentrations [7]. Alignment of the two protein sequences for bsPNP and hsPNP using BLAST from the NCBI website resulted in 87% identity at the amino acid level so it is highly possible that high Pi concentration may have a similar affect on hsPNP. It is not clear if the observed decrease in the potency of drug-inhibitors at high Pi concentration results from direct competition between the drug-inhibitor and the binding of Pi to the substrate binding site or from further hindrance of drug-inhibitor binding to the substrate binding site by an increase in Pi binding to the secondary Pi regulatory-site composed of Gln144 and Arg148 [5].

The goal of this investigation is to determine how the potency/binding affinity of three drug-inhibitors, acyclovir, ganciclovir, 9-benzylguanine that basically target the purine-binding site is affected by the presence of the second substrate, Pi, in its adjacent binding site. The binding thermodynamics of the three drug-inhibitors were determined from isothermal titration calorimetry (ITC) measurements as a function of the Pi concentration. The drug-inhibitors are guanine derivatives with different side-chains attached to the N9 position of the guanine ring as shown in Fig. 1: acyclovir has a simple acyclic 2-hydroxy-ethoxy-methyl side-chain, ganciclovir has a bulkier 2-hydroxy-1-methoxy-ethoxy-methyl side-chain, and 9-benzylguanine has benzyl ring for the side-chain. The inverse binding constants have been previously determined as inhibition constants (K_i) from enzyme inhibition assays employing Kalckar's assay [14]. Kalckar's assay follows the phosphorolysis catalyzed reaction by monitoring spectrophotometrically oxidation of the product, hypoxanthine, into uric acid in the presence of an enzyme, xanthine oxidase, subsequently added to the reaction mixture [14]. Acyclovir, an antiviral agent, is a weak competitive inhibitor of hsPNP with an inhibition constant $K_i = (91 \pm 9.7)$ μM [13,15]. Ganciclovir, a pro-drug nucleoside analog used in cancer research, possesses a 3-fold lower inhibition constant than acyclovir with a K_i value of 30 μM also at 50 mM Pi concentration and pH 7.4 [15]. 9-Benzylguanine exhibits the highest potency with the lowest inhibition constant of 7.0 ± 0.9 μM at 1 mM Pi concentration [16]. Recently reported ITC measurements on the binding of bsPNP to a acyclovir derivative where the $-CH_2-O-C_2H_4-OH$ side-chain is replaced by a longer phosphonate side-chain, $-CH_2-CH(CH_3)-O-CH_2-PO_3$, exhibited a more than two-fold increase in the binding enthalpy in the presence of 0.35 mM Pi without any change in the binding affinity [17]. An X-ray crystal structure of this complex also showed that in contrast to Pi, the phosphonate group re-directs the side-chain of Arg84 away from the binding side and, thus, lowers the expected inhibitory potency of the drug-inhibitor [17]. In addition, the specific