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Substrate specificity and kinetic mechanism of purine nucleoside phosphorylase from *Mycobacterium tuberculosis* ☆

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ABSTRACT

Purine nucleoside phosphorylase from *Mycobacterium tuberculosis* (MtPNP) is numbered among targets for persistence of the causative agent of tuberculosis. Here, it is shown that MtPNP is more specific to natural 6-oxopurine nucleosides and synthetic compounds, and does not catalyze the phosphorolysis of adenosine. Initial velocity, product inhibition and equilibrium binding data suggest that MtPNP catalyzes 2′-deoxyguanosine (2dGuo) phosphorolysis by a steady-state ordered bi bi kinetic mechanism, in which inorganic phosphate (P_i) binds first followed by 2dGuo, and ribose 1-phosphate dissociates first followed by guanine. pH-rate profiles indicated a general acid as being essential for both catalysis and 2dGuo binding, and that deprotonation of a group abolishes P_i binding. Proton inventory and solvent deuterium isotope effects indicate that a single solvent proton transfer makes a modest contribution to the rate-limiting step. Pre-steady-state kinetic data indicate that product release appears to contribute to the rate-limiting step for MtPNP-catalyzed reaction.

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Tuberculosis (TB)[1] remains a serious health threat worldwide, even though there are effective drugs [1] and an efficient chemotherapeutic treatment [2] against its causative agent, *Mycobacterium tuberculosis*. The emergence of acquired immunodeficiency syndrome (AIDS) caused by human immunodeficiency virus (HIV) infection and the poor access to drugs in developing nations [3] are among the factors that contribute to our failure to control the disease. Moreover, the Center of Disease Control and Prevention of USA has reported the worldwide emergence of extensively drug-resistant (XDR) TB cases, defined as cases in persons with TB whose isolates are multidrug-resistant as well as resistant to any one of the fluoroquinolone drugs and to at least one of the three injectable second-line drugs, amikacin, kanamycin or capreomycin [4,5]. Clinical manifestations have shown that XDR-TB is associated with greater morbidity and mortality than non-XDR-TB [6]. *M. tuberculosis* has been considered the world's most successful pathogen and this is largely due to its ability to persist in host tissues, where drugs that are rapidly bactericidal *in vitro* require prolonged administration to achieve comparable *in vivo* effects [7]. Approximately 2 billion individuals are believed to harbor latent TB [3], and its resumption of growth in a proportion of individuals sustains the pandemic of active TB [8]. Hence, more effective and less toxic anti-tubercular agents are needed to shorten the duration of current treatment, improve the treatment of drug-resistant TB, and to provide effective treatment of latent TB infection.

Long-term survival of nonreplicating *M. tuberculosis* is ensured by the coordinated shutdown of active metabolism through a broad transcriptional program called the stringent response. The synthesis and degradation of guanosine 3′,5′-bis(diphosphate) (ppGpp)

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[1] *Abbreviations used:* TB, tuberculosis; AIDS, acquired immunodeficiency syndrome; HIV, human immunodeficiency virus; XDR, extensively drug-resistant; ppGpp, guanosine 3′,5′-bis(diphosphate); PNP, purine nucleoside phosphorylase; MtPNP, PNP from *Mycobacterium tuberculosis*; ImmH, immucillin-H; SPR, surface plasmon resonance; Guo, guanosine; 7mGuo, 7-methylguanosine; Ino, inosine; 2dIno, 2′-deoxyinosine; Gua, guanine; 2dGuo, 2′-deoxyguanosine; R1P, ribose 1-phosphate; MESG, 2-amino-6-mercapto-7-methylpurine ribonucleoside; HPLC, high performance liquid chromatography; FPLC, fast performance liquid chromatography; LB, Luria–Bertani; IPTG, isopropyl β-D-thiogalactopyranoside; Tris, tris(Hydroxymethyl)aminomethane; MWCO, molecular weight cutoff; SDS–PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; 7mGua, 7-methylguanine; P_i, inorganic phosphate; MES, 2-(*N*-morpholino)ethanesulfonic acid; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; RU, response units; HsPNP, human PNP.

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and pppGpp are catalyzed by (p)ppGpp synthase I (*relA*, Rv2583) using GTP as substrate [9]. Increased concentration of hyperphosphorylated guanosine moieties is a central feature of pleiotropic physiological response of the stringent response, through which bacteria enter a latent state in response to nutritional stress [10]. The accumulation of ppGpp has been implicated in the latency of *Mycobacterium smegmatis* [11]. A *rel*-deficient mutant of *M. tuberculosis* was shown no longer be capable of ppGpp synthesis and this mutant had impaired long-term survival during *in vitro* starvation, indicating that ppGpp concentration may control mycobacterial adaptation to growth-limiting condition, allowing for long-term survival [12]. It has been shown that the *rel*-deficient *M. tuberculosis* mutant strain has impaired ability to sustain chronic infection in a murine model of persistent TB [13]. More recently, a comparative genomic analysis between *M. tuberculosis* H37Ra (avirulent) and H37Rv (virulent) identified, among other changes, a missense mutation in a regulatory protein of stringent response [14]. In the *de novo* synthesis of purine ribonucleotides, the formation of AMP and GMP from IMP is irreversible, but purine bases, nucleosides, and nucleotides can be interconverted through the activities of purine nucleoside phosphorylase (PNP; *deoD*, Rv3307), adenosine deaminase (*add*, Rv3313c), and hypoxanthine–guanine phosphoribosyl transferase (*htp*, Rv3624c). We have previously suggested that inhibition of *M. tuberculosis* PNP (MtPNP) could potentially lead to the accumulation of guanine nucleotides since a putative guanylate kinase (*gmk*, Rv1389) and nucleoside diphosphate kinase (*ndkA*, Rv2445c) are encoded in the genome of *M. tuberculosis* [15]. Accordingly, the *deoD* gene product (PNP) has been numbered among the top 100% persistence targets by the TB Structural Genomics Consortium (www.webtb.org). However, the physiological role of PNP in *M. tuberculosis* remains to be demonstrated.

PNP plays a key role in purine salvage pathway. PNP (EC 2.4.2.1) catalyzes the reversible phosphorolysis of *N*-glycosidic bond of β-purine (deoxy)ribonucleosides to generate α-(deoxy)ribose 1-phosphate and the corresponding purine bases [16,17] (Fig. 1). Immucillin-H (ImmH), a transition state analogue, has been shown to be a slow-onset inhibitor of MtPNP with an overall inhibition constant in the pM range [15]. The three-dimensional structure of MtPNP:ImmH:P_i ternary complex showed that the edge of the phenolic ring in Tyr188 is oriented perpendicular to the plane of the ImmH base and also forms a 2.8 Å hydrogen bond with the 5′-hydroxyl group of the transition state analogue [18]. Tyr188 is the only residue that interacts directly with bound inhibitor that is not conserved in the mammalian PNPs and it is thus likely that 5′-modifications of ImmH may yield inhibitors more specific for MtPNP. In addition, it has recently been shown that even though bovine and human PNPs share 87% sequence identity and have totally conserved active site residues, inhibitors with differential specificity can be designed [19].

Here, we describe MtPNP substrate specificity, steady-state kinetic parameters, product inhibition, pH-rate profiles, energy of activation, solvent kinetic isotope effects and proton inventory, equilibrium fluorescence spectroscopy upon binary and ternary complex formation, surface plasmon resonance (SPR) measurements upon binary complex formation, and pre-steady-state kinetics of product formation. The results described here should be useful to the design of MtPNP inhibitors with potential action against *M. tuberculosis* growth.

Materials and methods

Materials

All chemicals used were of analytical or reagent grade and required no further purification. Adenosine, guanosine (Guo), 7-methylguanosine (7mGuo), inosine (Ino), 2′-deoxyinosine (2dIno), guanine (Gua), and lysozyme were from Sigma. Adenine was from MP Biomedicals. 2′-Deoxyguanosine (2dGuo) and ribose 1-phosphate (R1P) were from Fluka BioChemika. 2-Amino-6-mercapto-7-methylpurine ribonucleoside (MESG) is commercially available in the Enzchek phosphate assay kit from Molecular Probes. Complete protease inhibitor cocktail tablets were from Boehringer (Mannheim, Germany). Protein assay kit was from Bio-Rad. Deuterium oxide (99.9 atom% D_2O) was from Cambridge Isotope Laboratories. Amicon stirred ultrafiltration cell, regenerated cellulose ultrafiltration membranes, and Centricon centrifugal filter devices were all from Millipore. High performance liquid chromatography (HPLC) assays (25 °C) and fast performance liquid chromatography (FPLC) protein purification (4 °C) were carried out in an Äkta purifier from GE Helthcare; all chromatographic columns were also from GE Helthcare. All steady-state activity assays were carried out in an UV-2550 UV–Visible Spectrophotometer (Shimadzu), and fluorescence binding experiments were carried out in a RF-5301PC Spectrophotometer (Shimadzu). SPR measurements were carried out in a BIA-Core *X* equipment using a carboxymethyl-dextran coated gold surface (Sensor Chip CM5), both from Biacore AB. Pre-steady-state measurements were performed using an Applied Photophysics (London, UK) SX.18MV-R stopped-flow spectrofluorimeter on absorbance mode.

Recombinant enzyme expression and purification

Recombinant MtPNP expression was as previously described [15], with some modifications. The pET-23a(+)::*deoD* recombinant expression plasmid [15] was transformed into *Escherichia coli* BL21(DE3)NH [20] competent cells. The cells were grown in Luria–Bertani (LB) medium pH 7.2 containing 50 μg mL^{-1} ampicillin at 37 °C to an OD_{600nm} of 0.5, induced by 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG) [21], and allowed to grow for additional 50.7 h. All subsequent steps were performed at 4 °C, unless stated otherwise. Approximately 20 g of cells were harvested by centrifugation (6000*g* for 30 min) from 9 L of LB media, washed with 50 mM tris(Hydroxymethyl)aminomethane (Tris) pH 7.6, and stored at −20 °C. Frozen cells (20 g) were thawed and suspended in 40 mL of 50 mM Tris pH 7.6 (buffer A1) containing a cocktail of protease inhibitors (Complete) and 0.2 mg mL^{-1} lysozyme, and the mixture was stirred for 30 min. Cells were disrupted by sonication, and centrifuged (40,000*g* for 1 h) to remove cell debris. The supernatant was incubated with 1% streptomycin sulfate (final concentration) for 40 min to precipitate nucleic acids and

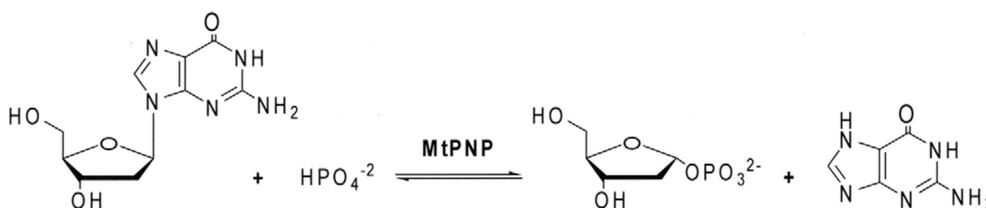

Fig. 1. Reaction catalyzed by MtPNP with 2′-deoxyguanosine, a natural substrate.

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