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UMP kinase from *Mycobacterium tuberculosis*: Mode of action and allosteric interactions, and their likely role in pyrimidine metabolism regulation ☆

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ABSTRACT

The *pyrH*-encoded uridine 5′-monophosphate kinase (UMPK) is involved in both *de novo* and salvage synthesis of DNA and RNA precursors. Here we describe *Mycobacterium tuberculosis* UMPK (*Mt*UMPK) cloning and expression in *Escherichia coli*. N-terminal amino acid sequencing and electrospray ionization mass spectrometry analyses confirmed the identity of homogeneous *Mt*UMPK. *Mt*UMPK catalyzed the phosphorylation of UMP to UDP, using ATP–Mg^{2+} as phosphate donor. Size exclusion chromatography showed that the protein is a homotetramer. Kinetic studies revealed that *Mt*UMPK exhibits cooperative kinetics towards ATP and undergoes allosteric regulation. GTP and UTP are, respectively, positive and negative effectors, maintaining the balance of purine versus pyrimidine synthesis. Initial velocity studies and substrate(s) binding measured by isothermal titration calorimetry suggested that catalysis proceeds by a sequential ordered mechanism, in which ATP binds first followed by UMP binding, and release of products is random. As *Mt*UMPK does not resemble its eukaryotic counterparts, specific inhibitors could be designed to be tested as antitubercular agents.

Introduction

Human tuberculosis (TB),[1] mainly caused by *Mycobacterium tuberculosis*, is a major cause of illness and death worldwide. *M. tuberculosis* is a remarkably successful pathogen that latently infects one third of the world population [1] and, despite the availability of effective chemotherapy and moderately protective vaccine, the tubercle bacillus continues to claim more lives than any other single infectious agent [2]. Increasing HIV–TB co-infections [2], the emergence of multidrug-resistant (MDR), extensively drug-resistant (XDR) [3], and, more recently, of totally drug-resistant strains (TDR) [4] have highlighted the need for the development of new therapeutic strategies to combat TB. Strategies based on the discovery of new targets for antimycobacterial agent development include elucidation of

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[1] *Abbreviations used:* ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; CMP, cytosine 5′-monophosphate; CTP, cytosine 5′-triphosphate; dCMP, deoxycytosine 5′-monophosphate; DMSO, dimethyl sulfoxide; DNA, deoxyribonucleic acid; dTMP, deoxythymidine 5′-monophosphate; ESI-MS, electrospray ionization mass spectrometry; FDA, Food and Drug Administration; GTP, guanosine 5′-triphosphate; Hepes, *N*-2-hydroxyethylpiperazyne-*N*′-2-ethanesulfonic acid; HIV, human immunodeficiency virus; IPTG, isopropyl-β-D-thiogalactopyranoside; ITC, isothermal titration calorimetry; LB, Luria–Bertani; MDR, multidrug-resistant; *Mt*UMPK, uridine 5′-monophosphate kinase from *Mycobacterium tuberculosis*; NADH, nicotinamide adenine dinucleotide; NDP, nucleoside diphosphate; NMP, nucleoside monophosphate; NTP, nucleoside triphosphate; PCR, polymerase chain reaction; PDB, Protein Data Bank; RNA, ribonucleic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TB, Terrific Broth; TB, tuberculosis; Tris, tris(hydroxymethyl)aminomethane; UDP, uridine 5′-diphosphate; UMP, uridine 5′-monophosphate; UMPK, uridine 5′-monophosphate kinase; UTP, uridine 5′-triphosphate; XDR, extensively drug-resistant.

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the role played by proteins of essential and, preferentially, exclusive biochemical pathways for mycobacterial growth [5].

Rational inhibitor design relies on mechanistic and structural information on the target enzyme. Enzyme inhibitors make up roughly 25% of the drugs marketed in United States [6]. Enzymes offer unique opportunities for drug design that are not available to cell surface receptors, nuclear hormone receptors, ion channel, transporters, and DNA [6]. It has been pointed out that one of the lessons to be learned from marketed enzyme inhibitors is that the most potent and effective inhibitors take advantage of enzyme chemistry to achieve inhibition [7]. Moreover, the recognition of the limitations of high-throughput screening approaches in the discovery of candidate drugs has rekindled interest in rational design methods [8]. Accordingly, mechanistic analysis should always be a top priority for enzyme-targeted drug programs aiming at the rational design of potent enzyme inhibitors.

Nucleotides are important molecules present in all living organisms as they constitute the building blocks for nucleic acids and also serve as energy sources for many biochemical reactions [9]. Pyrimidine nucleotides can be synthesized by *de novo* and salvage pathways resulting in a common product, the nucleotide uridine 5′-monophosphate (UMP) [10]. Subsequent phosphorylation of UMP yields UDP that leads to the synthesis of all other pyrimidine nucleotides [11]. Nucleoside monophosphate (NMP) kinases play an important role in the biosynthesis of nucleotides and represent a homogeneous family of catalysts related to adenylate kinase (EC 2.7.4.3). They catalyze the synthesis of nucleoside diphosphates (NDPs), which will be converted to nucleoside triphosphates (NTPs) by a non-specific nucleoside diphosphate kinase [12]. UMP kinases (UMPKs) catalyze the reversible transfer of the γ-phosphoryl group from ATP to UMP in the presence of a divalent cation, usually Mg^{2+} (Fig. 1) [13]. In general, eukaryotic UMP/CMP kinases (EC 2.7.4.14) are monomers, phosphorylate with comparable efficiency both UMP and CMP, and are structurally similar to other NMP kinases (such as adenylate kinase) [12,14–16]. In contrast, bacterial UMPKs (EC 2.7.4.22) are specific for UMP, exist in solution as stable homohexamers, and do not resemble either UMP/CMP kinases or NMP kinases from other organisms based on sequence comparisons [17,18]. Kinetic studies have shown that bacterial UMPKs can be activated by GTP and/or be subject to feedback inhibition by UTP, the major product of the reaction they catalyze [17–21], regulating the balance of purine versus pyrimidine nucleoside triphosphates synthesis [13].

As pyrimidine biosynthesis is an essential step in the progression of TB, enzymes of this pathway are attractive antitubercular drug targets [22]. Homologs to enzymes in the pyrimidine pathway have been identified in the genome sequence of *M. tuberculosis* [23]. A rapid recombination method for screening and confirmation of gene essentiality has recently been proposed to allow identification of which of the approximately 4000 genes of *M. tuberculosis* are worthy of further study as drug targets [24]. The product of *pyrH* (Rv2883) gene has been shown to be essential for *M. tuberculosis* growth by the rapid screening method [24].

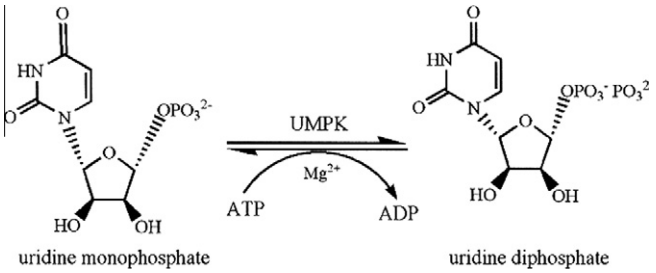

Fig. 1. Chemical reaction catalyzed by UMPK.

Genetic studies have provided evidence that UMPK is essential for growth in both Gram-negative (*Escherichia coli*) [25,26] and Gram-positive bacteria (*Streptococcus pneumoniae*) [19]. Although the *pyrH* gene has been proposed by sequence homology to encode a UMPK protein [23], there has been no formal biochemical proof as to ascertain the correct assignment to the open reading frame of *pyrH* gene in *M. tuberculosis*.

In the present work, the *pyrH* gene from *M. tuberculosis* strain H37Rv was PCR amplified, cloned, and recombinant UMPK (*Mt*UMPK) was purified to homogeneity. N-terminal amino acid sequencing and electrospray ionization mass spectrometry (ESI-MS) analyses were carried out to confirm the identity of the recombinant *Mt*UMPK protein. Initial velocity studies were performed to evaluate the kinetic parameters of the recombinant *Mt*UMPK. In addition, isothermal titration calorimetry study of substrates binding was carried out to demonstrate the order of substrate addition in the kinetic mechanism of *Mt*UMPK. Protein allosteric regulation by ATP, GTP, and UTP have also been demonstrated. These results represent an important step for the rational design of *Mt*UMPK inhibitors that can further be tested as anti-TB drugs.

Materials and methods

Amplification, cloning and DNA sequencing of the pyrH gene

The full-length *pyrH* (Rv2883c) coding region [23] was PCR amplified using the genomic DNA from *M. tuberculosis* H37Rv as template and a high fidelity proof-reading thermostable DNA polymerase (*Pfu*® DNA polymerase, Stratagene). The synthetic oligonucleotides used (forward primer, 5′-GTC ATA TGA CAG AGC CCG ATG TCG CCG GC-3′; and reverse primer, 5′-TAA AGC TTT CAG GTG GTG ACC AGC GTT CCG A-3′) were designed to contain, respectively, NdeI and HindIII (New England Biolabs) restriction sites (underlined). Dimethyl sulfoxide (DMSO) was added to a final concentration of 10%. The 786-bp amplicon was detected on 1% agarose gel and purified utilizing the Quick Gel Extraction kit (Invitrogen). The PCR fragment was cloned into pCR-Blunt® vector (Invitrogen) and, following transformation of *E. coli* strain DH10B (Novagen), the resulting plasmid was isolated utilizing the Qiaprep Spin Miniprep kit (Qiagen). Subsequently, the fragment was cleaved with NdeI and HindIII endonucleases and inserted into the pET-23a(+) expression vector (Novagen), previously digested with the same restriction enzymes. The complete *pyrH* nucleotide sequence was determined by automated DNA sequencing to corroborate sequence identity, integrity and to check the absence of mutations in the cloned fragment.

Expression and purification of recombinant MtUMPK

The recombinant plasmid pET-23a(+)::*pyrH* was transformed into BL21(DE3) *E. coli* electrocompetent cells (Novagen), and cells carrying the recombinant vector were selected on Luria–Bertani (LB) agar plates containing 50 μg mL^{-1} ampicillin [27]. A single colony was used to inoculate 50 mL of Terrific Broth (TB) medium containing the same antibiotic and grown overnight at 37 °C. Aliquots of this culture (2.5 mL) were used to inoculate 250 mL of TB medium in 5 × 1 L flasks supplemented with ampicillin (50 μg mL^{-1}) and grown at 37 °C and 180 rpm to an optical density (OD_{600nm}) of 0.4–0.6. When this OD_{600} value was reached, the temperature was lowered to 30 °C and protein expression was carried out without isopropyl-β-D-thiogalactopyranoside (IPTG) induction. After 24 h, the cells (12 g) were collected by centrifugation at 11,800*g* for 30 min at 4 °C and stored at −20 °C. The same protocol was employed for BL21 (DE3) *E. coli* cell transformed with pET-23a(+) as control. The expression of the recombinant protein was confirmed by 12% sodium