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Kinetic mechanism and energetics of binding of phosphoryl group acceptors to *Mycobacterium tuberculosis* cytidine monophosphate kinase

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ABSTRACT

Cytidine monophosphate kinase from *Mycobacterium tuberculosis* (*Mt*CMK) likely plays a role in supplying precursors for nucleic acid synthesis. *Mt*CMK catalyzes the ATP-dependent phosphoryl group transfer preferentially to CMP and dCMP. Initial velocity studies and Isothermal titration calorimetry (ITC) measurements showed that *Mt*CMK follows a random-order mechanism of substrate (CMP and ATP) binding, and an ordered mechanism for product release, in which ADP is released first followed by CDP. The thermodynamic signatures of CMP and CDP binding to *Mt*CMK showed favorable enthalpy and unfavorable entropy, and ATP binding was characterized by favorable changes in enthalpy and entropy. The contribution of linked protonation events to the energetics of *Mt*CMK:phosphoryl group acceptor binary complex formation suggested a net gain of protons. Values for the pK_a of a likely chemical group involved in proton exchange and for the intrinsic binding enthalpy were calculated. The Asp187 side chain of *Mt*CMK is suggested as the likely candidate for the protonation event. Data on thermodynamics of binary complex formation were collected to evaluate the contribution of 2′-OH group to intermolecular interactions. The data are discussed in light of functional and structural comparisons between CMP/dCMP kinases and UMP/CMP ones.

Introduction

Human tuberculosis (TB)[2], caused mainly by *Mycobacterium tuberculosis*, remains one of the most critical problems for global health control, reaching over 1.4 million deaths in 2010 [1–3]. TB "short-course" therapy consists of 2 months of treatment with rifampicin, isoniazid, ethambutol and pyrazinamide, followed by 4 months of treatment with isoniazid and rifampicin [3]. However, noncompliance with TB treatment gives rise to appearance of drug-resistant strains of *M. tuberculosis* [4–6], which underlines the need for alternative therapeutic chemical entities to both shorten the duration of therapy and combat the problem of clinical drug resistance. As the *cmk*-gene product has been predicted to be required for optimal *in vitro* growth of *M. tuberculosis* H37Rv strain by Himar1-based transposon mutagenesis [7]. More recently, high-resolution global phenotypic profiling results have prompted the proposal that the *cmk*-gene product is essential for *in vitro* growth in glycerol and cholesterol (a critical carbon source during infection) media [8]. Accordingly, efforts to understand the mode of action of the *cmk*-gene product appear to be worth pursuing to provide a basis

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[2] *Abbreviations used:* ADP, adenosine 5′-diphosphate; AraCMP, cytosine-β-D-arabinofuranoside 5-monophosphate; ATP, adenosine 5′-triphosphate; CDP, cytidine 5′-diphosphate; CHES, cyclohexylaminoethanesulfonic acid; CMP, cytidine 5′-monophosphate; CMK, cytidine 5-monophosphate kinase; dCMP, 2-deoxy-cytidine 5-monophosphate; ddCMP, 2-3-dideoxy-cytidine 5-monophosphate; ESI-MS, electrospray ionization mass spectrometry; HEPES, *N*-2-hydroxyethylpiperazyne-*N*′-2-ethanesulfonic acid; HEPPS, *N*-2-hydroxyethylpiperazine-*N*′-3-propane-sulfonic acid; ITC, isothermal titration calorimetry; LDH, lactate dehydrogenase; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfinic acis; MtCMK, cytidine monophosphate kinase from *Mycobacterium tuberculosis*; NADH, reduced nicotinamide adenine dinucleotide; NMP, nucleoside monophosphate; NMP kinases, nucleoside monophosphate kinases; PEP, phosphoenolpyruvate; PK, pyruvate kinase; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TB, tuberculosis; Tris, tris(hydroxymethyl)aminomethane; UMP, uridine 5-monophosphate; UMPK, uridine 5-monophosphate kinase.

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for function-based design of inhibitors with potential anti-tubercular activity.

Nucleoside monophosphate (NMP) kinases play pivotal roles in both *de novo* synthesis and salvage pathway of DNA and RNA precursors [9,10]. Bacterial cytidine 5′-monophosphate (CMP) kinase (CMK), which is part of pyrimidine nucleotide interconversion pathways, catalyzes the γ-phosphoryl group transfer from, usually, adenosine-5′-triphosphate (ATP) to either CMP or 2′-deoxy-CMP (dCMP). We have previously reported that *cmk* gene in *M. tuberculosis* encodes a protein with CMK activity (*Mt*CMK), which catalyzes the phosphoryl transfer from ATP to dCMP, CMP or uridine 5′-monophosphate (UMP) to form the corresponding nucleoside diphosphates [11]. However, *Mt*CMK preferentially phosphorylates dCMP and CMP, whereas UMP is a poor substrate [11] (Fig. 1). The specificity of *Mt*CMK for dCMP and CMP is not surprising since it has recently been shown that the Rv2883c locus (*pyrH*) in *M. tuberculosis* codes for a functional uridine 5′-monophosphate kinase (*Mt*UMPK) [12].

Bacterial CMP kinases are regarded as potential drug targets due to the primary structure divergence and substrate affinity when compared with the human orthologue UMP/CMP kinase [13]. In addition, CMK enzymes have been reported to be essential for different organisms: *Streptococcus pneumonia* [14], *Bacillus subtilis* [15], *Salmonella typhimurium* [16] and, probably, *M. tuberculosis* [7,8] due to its proposed role in recycling nucleotides derived from RNA degradation in the latter [11].

NMP kinases undergo large conformational changes upon binding of substrates [17]. NMP kinases have an overall well-conserved fold with three domains [18]: the relatively rigid CORE domain (containing a five-stranded parallel β-sheet), the LID domain that closes the active site upon ATP binding, and the NMP binding domain, which closes the active site upon binding of the phosphate acceptor. NMP kinases are divided into short and long forms. The latter group consists of adenylate kinases with an insertion of approximately 27 amino acid residues in the LID domain [13]. Bacterial CMKs, including *Mt*CMK, belongs to a third family of NMP kinases as they possess distinct features: a short LID domain, and an insertion of 40 amino acid residues in the NMP-binding domain [18].

In the present work, isothermal titration calorimetry (ITC) studies were performed to determine the order, if any, of addition of substrates (CMP and ATP) to and release of products (CDP and ADP) from *Mt*CMK. Initial velocity studies were also carried out to evaluate the specificity of *Mt*CMK for phosphate acceptor using cytosine-β-D-arabinofuranoside 5′-monophosphate (AraCMP) and 2′-3′-dideoxy-cytidine 5′-monophosphate (ddCMP) as substrates. The number of protons released/taken up upon binary complex formation between *Mt*CMK and either CMP, dCMP or AraCMP were evaluated using buffers with different enthalpies of ionization. An estimate for the pK_a of a likely chemical group involved in proton exchange upon binary complex formation was obtained from pH-dependence of the binding energetics. A linkage between binary complex formation and protonation was observed and a value for the intrinsic enthalpy of binding was determined. Data on thermodynamics of CMP, dCMP and AraCMP binding to *Mt*CMK were collected to evaluate the contribution of 2′-OH group to binary complex formation. The data here presented provide a thermodynamic evaluation of the role of ionizable groups involved in *Mt*CMK:CMP, *Mt*CMK:dCMP and *Mt*CMK:AraCMP complex formation.

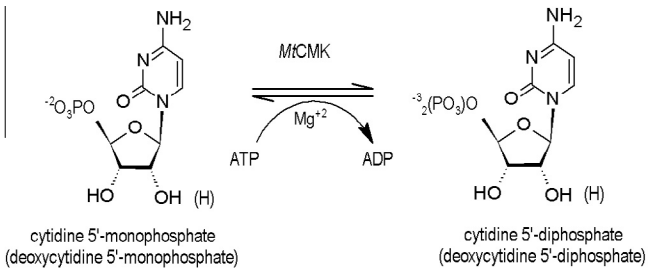

Fig. 1. Chemical reaction catalyzed by *Mt*CMK. This enzyme catalyzes the ATP-dependent phosphoryl group transfer preferentially to CMP and dCMP [11].

Materials and methods

Materials

Chemicals were of analytical or reagent grade and used without further purification. ATP, CMP, cytidine 5′-diphosphate (CDP) and dCMP were purchased from Sigma–Aldrich. AraCMP was obtained from Santa Cruz Biotechnology® and ddCMP from Synfine Research. All purification steps were carried out using an ÄKTA system (GE Healthcare) at 4 °C and sample elution was monitored by UV detection. Protein fractions were analyzed by SDS–PAGE 12% [19]. The steady-state activity assays were performed using a UV-2550 UV/Visible spectrophotometer (Shimadzu) in 1 cm pathlength cuvettes and assay mixtures of 500 μL final volume. ITC experiments were carried out using an iTC200 Microcalorimeter (MicroCal, Inc., GE Healthcare).

Protein preparation

Electrocompetent BL21(DE3) *Escherichia coli* cells were transformed with pET-23a(+)::*cmk* recombinant plasmid, and cell growth and purification steps of *MtCMK* were as described elsewhere [11]. The pooled fractions were dialyzed against 50 mM Tris–HCl pH 7.5, 50 mM KCl and 5 mM $MgCl_2$, concentrated using an AMICON (Millipore Corporation, Bedford, MA) ultrafiltration membrane (MWCO = 10 kDa), and stored at −80 °C. Total protein concentration was determined by the method of Bradford [20], using the Bio-Rad protein assay kit (Bio-Rad Laboratories) and bovine serum albumin as standard. As described elsewhere [11], a typical yield of 5 mg of homogeneous recombinant *Mt*CMK per liter of cell culture could be obtained.

Enzyme activity assays and determination of steady-state kinetic parameters

Enzyme activity was routinely measured using CMP and ATP as substrates and the reaction was started by the addition of recombinant *Mt*CMK. All enzyme activity measurements were carried out at 25 °C. The ADP product formation was coupled to chemical reactions catalyzed by pyruvate kinase (PK; EC 2.7.1.40) and lactate dehydrogenase (LDH; EC 1.1.1.27), and the decrease in absorbance at 340 nm ($\varepsilon_{\beta\text{-NADH}} = 6.22 \times 10^3\ M^{-1}\ cm^{-1}$) due to NADH oxidation was monitored as described elsewhere [11,21,22]. The enzyme assay mixture contained 40 mM Hepes pH 7.6, 40 mM KCl and 5 mM $MgCl_2$, 1.5 mM PEP, 0.2 mM NADH, 6 U mL^{-1} PK, and 5 U mL^{-1} LDH. Initial steady-state rates were calculated from the linear portion of the reaction curve under experimental conditions in which less than 5% of substrate was consumed.

To evaluate further the specificity for phosphate acceptor, CMP was replaced with either AraCMP or ddCMP (nucleoside monophosphate analogs), at varying concentrations, utilizing the coupled standard assay. Initially, increasing amounts of *Mt*CMK were added to the reaction mixture, and measurements of enzymatic reaction with the nucleotide analogue AraCMP (1 mM) and the substrate ATP (1 mM) were carried out to determine the range in which the enzyme activity is linearly dependent on recombinant enzyme concentration. The true steady-state kinetic parameters and initial