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Catalytic mechanism and cofactor preference of dihydrodipicolinate reductase from methicillin-resistant *Staphylococcus aureus*

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ABSTRACT

Given the rapid rise in antibiotic resistance, including methicillin resistance in *Staphylococcus aureus* (MRSA), there is an urgent need to characterize novel drug targets. Enzymes of the lysine biosynthesis pathway in bacteria are examples of such targets, including dihydrodipicolinate reductase (DHDPR, E.C. 1.3.1.26), which is the product of an essential bacterial gene. DHDPR catalyzes the NAD(P)H-dependent reduction of dihydrodipicolinate (DHDP) to tetrahydrodipicolinate (THDP) in the lysine biosynthesis pathway. We show that MRSA–DHDPR exhibits a unique nucleotide specificity utilizing NADPH (K_m = 12 μM) as a cofactor more effectively than NADH (K_m = 26 μM). However, the enzyme is inhibited by high concentrations of DHDP when using NADPH as a cofactor, but not with NADH. Isothermal titration calorimetry (ITC) studies reveal that MRSA–DHDPR has ~20-fold greater binding affinity for NADPH (K_d = 1.5 μM) relative to NADH (K_d = 29 μM). Kinetic investigations in tandem with ITC studies show that the enzyme follows a compulsory-order ternary complex mechanism; with inhibition by DHDP through the formation of a nonproductive ternary complex with $NADP^+$. This work describes, for the first time, the catalytic mechanism and cofactor preference of MRSA–DHDPR, and provides insight into rational approaches to inhibiting this valid antimicrobial target.

Introduction

Staphylococcus aureus is a Gram-positive facultative anaerobe pathogen that commonly colonizes the anterior nares, respiratory system, and urinary tract of the host [1]. The organism can also enter open wounds and is thus capable of causing multi-systemic life-threatening infections in humans [2]. Methicillin-resistant *S. aureus* (MRSA)[2] is a strain that has developed antibiotic resistance to all penicillin-based antibiotics, including methicillin [3]. Two major variants of MRSA have been described, namely, hospital-acquired MRSA (HA-MRSA) and community-acquired MRSA (CA-MRSA) [4]. Currently, all available β-lactam antibiotics are ineffective against HA-MRSA and CA-MRSA strains [5,6]. Therefore, vancomycin is employed as the antibiotic of last resort for treating MRSA infections, but reports of resistance to vancomycin are nonetheless emerging at a rapid rate [7,8]. As a result, the morbidity and mortality rates of MRSA infections are increasing worldwide [9], and there is thus an urgent need to discover new antibiotics for the treatment of MRSA infections and an equally urgent need to characterize novel antibiotic targets. One such target is the lysine biosynthesis pathway (also known as the diaminopimelate pathway) in bacteria [7,10]. The products of the pathway include *meso*-diaminopimelate and lysine, that are essential building blocks for the synthesis of the bacterial cell wall, housekeeping proteins, and virulence factors [7,10]. Accordingly, the enzymes that catalyze critical steps in the pathway serve as excellent antimicrobial targets [7,10–12]. This is further validated given that humans do not contain the enzymatic machinery used to synthesize lysine, instead acquiring this essential amino acid from dietary sources [7,10].

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[2] *Abbreviations used:* (*S*)-ASA, aspartate semi-aldehyde; DHDP, dihydrodipicolinate; DHDPR, dihydrodipicolinate reductase; DHDPS, dihydrodipicolinate synthase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ITC, isothermal titration calorimetry; MRSA, methicillin-resistant *Staphylococcus aureus*; NAD^+, nicotinamide adenine dinucleotide (oxidized); NADH, nicotinamide adenine dinucleotide (reduced); $NADP^+$, nicotinamide adenine dinucleotide phosphate (oxidized); NADPH, nicotinamide adenine dinucleotide phosphate (reduced); 2,6-PDC, 2,6-pyridine dicarboxylate; THDP, tetrahydrodipicolinate; *v*, initial velocity.

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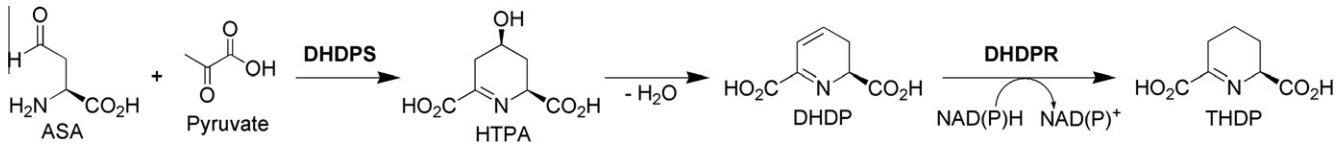

Fig. 1. Schematic representation of the lysine biosynthesis pathway showing the DHDPS and DHDPR catalyzed reactions.

In recent years there has been heightened interest in studying the enzymatic machinery of bacterial lysine biosynthesis, particularly in characterizing the kinetic properties, regulation, and three-dimensional structures of both wild-type and mutant enzymes [13–25]. Studies have also focused on determining the essentiality of the enzymes to bacteria [10]. For example, in a landmark study by Kobayashi et al. [26], the authors systematically knocked out all 4118 genes comprising the *Bacillus subtilis* genome and showed that only 271 genes were essential for viability, including the *dapB* gene that encodes the enzyme dihydrodipicolinate reductase (DHDPR).

The first committed step of the lysine biosynthesis pathway is catalyzed by dihydrodipicolinate synthase (DHDPS), which catalyzes the condensation of pyruvate and aspartate semi-aldehyde [(*S*)-ASA] forming the unstable heterocycle, HTPA (Fig. 1). HTPA is then non-enzymatically dehydrated to form dihydrodipicolinate (DHDP), which is subsequently reduced by DHDPR to form tetrahydrodipicolinate (THDP) using NAD(P)H as the reductant (Fig. 1) [27]. DHDPR has been characterized from a number of bacterial species, including *Bacillus cereus*, *Bacillus megaterium*, *B. subtilis*, *Corynebacterium glutamicum*, *Escherichia coli*, *Methylophilus methylotrophus*, *Mycobacterium tuberculosis*, and *Thermotoga maritima* [28–35]. The three-dimensional structure of this enzyme has been determined from *E. coli*, *M. tuberculosis* and *T. maritima* in the absence and/or presence of cofactor [29–31,36]. Like most nucleotide-dependent reductases, dual specificity and/or preference for one of the nucleotides NADH and/or NADPH has been reported for DHDPR from different organisms [28–30]. Moreover, DHDPR from *E. coli* and *M. tuberculosis* have been shown to have dual cofactor specificity, with *E. coli* DHDPR having a two-fold greater affinity for NADH over NADPH [28,31,32]. In contrast, DHDPR from *T. maritima* possesses significantly greater affinity for NADPH compared to NADH, but is inhibited by the latter at higher concentrations [29]. Whereas, DHDPR from *M. tuberculosis* is able to utilize both cofactors with equal efficiency [30].

Given that DHDPR from diverse bacterial species demonstrate different cofactor preferences, the aim of this study was to kinetically and thermodynamically characterize the enzymatic mechanism and cofactor preference of MRSA–DHDPR. The results presented in this study demonstrate that MRSA–DHDPR catalyzes the reduction of DHDP to THDP using a compulsory-order ternary complex mechanism with cofactor preference for NADPH. However, the enzyme displays substrate inhibition at high DHDP concentrations when using NADPH as the cofactor. This study offers important insight into rational drug design strategies for inhibiting a novel antibiotic target from methicillin-resistant *S. aureus*.

Materials and methods

Materials

NAD^+, NADH, $NADP^+$, NADPH, 2,6-pyridine dicarboxylate and sodium pyruvate, were purchased from Sigma–Aldrich (Australia), HEPES was obtained from Ajax Finechem Pty., Ltd. (Australia), and (*S*)-aspartate semi-aldehyde was synthesized as described in [37].

Cloning, expression and purification of MRSA–DHDPR and E. coli DHDPS

The *dapB* gene from MRSA strain 252 was amplified by PCR and cloned into the pET11a expression vector. Recombinant protein was produced in *E. coli* BL21 (DE3) cells, and purified to yield a >95% homogeneous enzyme preparation as described in [14]. Dihydrodipicolinate synthase (DHDPS) from *E. coli* (required for the coupled enzyme kinetic assay) was expressed and purified to yield >95% pure enzyme as described in [23].

Coupled enzyme kinetics assay

The coupled assay employing DHDPS and DHDPR [28,29] was measured at a wavelength of 340 nm at 30 °C using a 1 cm acrylic cuvette and a Cary 4000 spectrophotometer. The assay was performed in HEPES buffer, pH 8.0, at a final concentration of 100 mM with a final assay volume of 800 μl. The standard constituents of the assay include 70 nM MRSA–DHDPR (final assay concentration), an excess amount of *E. coli* DHDPS as the coupling enzyme, NADPH or NADH as the cofactor, (*S*)-aspartate semi-aldehyde [(*S*)-ASA], and pyruvate. *E. coli* DHDPS was considered to be in excess when addition of further amounts of the enzyme did not result in an increase to the initial reaction rate (i.e. final assay concentration of 1.6 μM). The assay mixture was incubated at 30 °C for approximately 3 min with *E. coli* DHDPS and initiated by the addition of MRSA–DHDPR. All assays were performed in duplicate to ensure that K_m and V_{max} values were reproducible. To determine the kinetic parameters for the substrate (i.e. DHDP), (*S*)-ASA concentration was varied from 0.025 mM to 1.0 mM, with pyruvate maintained at a constant concentration of 2 mM, and with the concentration of cofactor (NADH or NADPH) fixed at either a concentration of 20 μM, 40 μM, 60 μM, 80 μM or 100 μM. The coupled assay is accurate when using cofactor (i.e. NADH or NADPH) concentrations $\geqslant$20 μM and (*S*)-ASA concentrations $\geqslant$0.025 mM. Initial rates for MRSA–DHDPR using both NADH and NADPH were determined from the change in absorbance at 340 nm (ΔA_{340nm}) as a function of time using ε_{340} [NAD(P)H] = 6220 M^{-1} cm^{-1} over the linear portion of the A_{340nm} versus time profiles.

Kinetic data analysis

Data were analyzed by nonlinear regression using the program ENZFITTER (Biosoft, Cambridge, UK). Eq. (1) was employed to fit a compulsory-order ternary-complex mechanism, and Eq. (2) to fit a compulsory-order ternary-complex mechanism with substrate inhibition by DHDP.

$$v = (V_{max} * a * b)/((K_{iA} * K_{mB)} + (K_{mB} * a) + (K_{mA} * b) + (a * b)) \quad (1)$$

$$v = (V_{max} * a * b)/((K_{iA} * K_{mB}) + (K_{mB} * a) + (K_{mA} * b) + (a * b) * (1 + (b/K_{siB}))) \quad (2)$$

where v is the initial velocity, a is the co-factor [NAD(P)H] concentration, b is the substrate (DHDP) concentration, V_{max} is the limiting maximal velocity/rate, K_{mB} and K_{mA} are the Michaelis-Menten constants for substrates a and b, respectively, K_{iA} is the inhibition