Structure and nucleotide specificity of *Staphylococcus aureus* dihydrodipicolinate reductase (DapB)

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**Abstract**

Lysine biosynthesis proceeds by the nucleotide-dependent reduction of dihydrodipicolinate (DHDP) to tetrahydrodipicolinate (THDP) by dihydrodipicolinate reductase (DHDPR). The *S. aureus* DHDPR structure reveals different conformational states of this enzyme even in the absence of a substrate or nucleotide-cofactor. Despite lacking a conserved basic residue essential for NADPH interaction, *S. aureus* DHDPR differs from other homologues as NADPH is a more preferred co-factor than NADH. The structure provides a rationale—Lys35 compensates for the co-factor site mutation. These observations are significant for bi-ligand inhibitor design that relies on ligand-induced conformational changes as well as co-factor specificity for this important drug target.

**Keywords:**
DHDPR
DapB
MRS
Lysine/m-Dap biosynthesis

1. Introduction

*Staphylococcus aureus* is a pathogen responsible for superficial infections on skin and soft tissues and severe life threatening invasive infections like pneumonia, osteomyelitis, arthritis, endocarditis and sepsis [1,2]. *Staphylococci* can rapidly acquire resistance to several front-line antibiotics in both hospitals as well as community settings. Antibiotic resistance in these cocci involves very few mutations that distinguish between susceptible and the resistant strains [3]. In addition to the development of methicillin resistant (MRSA) strains, the recent emergence of vancomycin-resistant strains of *S. aureus* (VRSA), underscores the need to characterize novel drug targets from this evasive pathogen [4]. Enzymes of bacterial L-lysine/m-dap pathway are significant in this regard as they represent potential alternate targets that are absent in humans and have not been exploited in the treatment of *Staphylococcal* infections [5,6].

Dihydrodipicolinate reductase (DHDPR), a product of an essential gene referred to as *dapB*, catalyzes the second step of lysine biosynthesis. DHDPR catalyzes the reduction of 2,3-dihydrodipicolinate (DHDP) to 2,3,4,5-tetrahydrodipicolinate (THDP) in a nucleotide dependent reaction [7]. THDP is the common precursor for the biosynthesis of m-DAP/L-Lys in all characterized biosynthetic routes viz., the succinylase, acetylase and dehydrogenase pathways [8]. Recent reports, based on the characterization of the substrate specificity of dihydrodipicolinate synthase (DHDPS) revealed that DHDPR could also function as a dehydratase in addition to the role of a nucleotide dependent reductase [9]. Structures of DHDPR homologues from *Escherichia coli*, *Mycobacterium tuberculosis*, *Thermotoga maritima* and *Bartonella henselae* have been determined ([10,11,18]; PDB:1VM6 (Joint Centre for Structural Genomics) and PDB:3JP (Seattle Structural Genomics Center for Infectious Disease). DHDPR is a homotetramer with each monomer unit consisting of an N-terminal nucleotide binding (cofactor) domain and a C-terminal substrate binding (tetramerization) domain linked by a flexible loop. The active site is located at the interface between the nucleotide binding and substrate binding domains. Hydrogen exchange experiments [12] and the crystal structures of DHDPR bound to nucleotides and substrate analogues suggest that the hydride transfer reaction requires the movement of N-terminal domain towards the C-terminal domain [11,13].

DHDPR enzymes show large differences in their nucleotide binding specificity. Both *E. coli* DHDPR (EcDHDPR) and *M. tuberculosis* DHDPR (MtDHDPR) show dual nucleotide specificity with a preference for NADH over NADPH. The *T. maritima* enzyme (TmDHDPR), on the other hand, has higher affinity for NADPH over NADH. The dual specificity of EcDHDPR and MtDHDPR enzymes could be rationalized based on their crystal structures and sequence analysis [11,13,14]. The dual specificity of EcDHDPR could be attributed primarily to the interaction of Glu38 and Arg39 with bound nucleotides. Glu38 interacts with both 2'- and
3′-hydroxyl groups of the adenosyl ribose of NADH, while the adjacent Arg39 residue interacts with the 2′-phosphate of NADPH [13]. In the absence of a conserved basic residue equivalent to Arg39, Lys9 and Lys11 were proposed to indirectly stabilize the 2′-phosphate of NADPH in MtDHDPR [13]. However these residues are not conserved across all bacteria. In fact, both Lys9 and Lys11 (MtDHDPR and Arg39 (EcDHDPR) are not conserved in S. aureus DHDPR (SaDHDPR). Here we report the crystal structure and biochemical characterization of SaDHDPR that reveals dual specificity for nucleotide co-factors with a preference that differs from the E. coli and M. tuberculosis homologues. The structure of SaDHDPR at high resolution reveals distinctive features that could be employed for the design of inhibitors against this essential protein.

2. Materials and methods

2.1. Cloning, expression and purification of SaDHDPR

The dapB gene was PCR amplified from the genomic DNA of S. aureus COL and cloned into pET expression vectors (15b and 22b). These plasmid(s) were transformed into E. coli BL21(DE3) cells. The expression protocol for SaDHDPR and the K35A mutant was similar-transfomed cells were grown at 37 °C to an optical density of 0.6 at 600 nm. The cells were subsequently induced with 0.5 mM IPTG. Post induction, the cells were further grown at 21 °C for 5–6 h. The recombinant protein with a hexa-histidine tag at either the N- or the C-terminus was purified by Ni2+-NTA affinity chromatography. In the case of the recombinant DHDPR without a hexa-histidine tag, the cell-free lysate was passed through a DEAE-Sephrose column (3 ml of resin) at a flow rate of 0.3 ml/min. This partially purified protein was further purified by size exclusion chromatography.

2.2. Analysis of the quaternary association

An analytical size exclusion experiment was performed to ascertain the quaternary structure of S. aureus DHDPR in solution. About 100 µl of purified protein (2.0 mg/ml) was passed through a Superdex S-200 (10/300 GL) column at a flow rate of 0.3 ml/min equilibrated in 25 mM Tris–HCl pH 7.5 and 200 mM NaCl. Dynamic light scattering studies were carried out on a Dynapro Light Scattering instrument (Wyatt Technology Co., USA) at 2.0 mg/ml protein concentration in a buffer containing 50 mM Tris–HCl pH 7.5 and 200 mM NaCl.

2.3. Isothermal titration calorimetry

The purified SaDHDPR and the K35A mutant proteins were analyzed for their cofactor specificity by isothermal titration calorimetry (ITC). These experiments were performed in a VP-ITC MicroCalorimeter (MicroCal, Inc.) at 25 °C. Purified SaDHDPR samples were dialyzed against 25 mM HEPES pH 7.5 for 24 h at 4 °C prior to the ITC experiment. The sample cell (1.4 ml) had 75 µM of protein and the syringe (volume 298 µl) contained 2 mM of the nucleotide (NADH/NADPH) solution. Titrations were performed by a step-wise addition of 5 µl ligand solution into the sample cell in 8 s. A time interval of 180 s between successive injections and a stirring speed of 351 rpm was maintained throughout the titration. The data could be best fitted to an equation describing the ligand binding to a macromolecule possessing a set of independent binding sites (Origin software).

2.4. Crystallization and structure determination

Crystallization trials of SaDHDPR involved different recombinant protein constructs and optimization steps (Supplementary methods 1). Crystallization experiments with SaDHDPR without a hexa-histidine tag yielded a single crystal in a condition containing 2.0 M ammonium sulfate and 0.2 M sodium acetate pH 4.5. Diffraction data were collected on a CCD detector with 0.5° oscillation per image at the beam-line BM14 of the European synchrotron radiation facility (ESRF). The data were processed using MOSFLM [15] and scaled using SCALA [16]. The data collection and refinement statistics are compiled in Table 1. The molecular replacement (MR) trials using the intact polypeptide chain of either EcDHDPR (PDB:1ARZ) or TmDHDPR (PDB:1VM6) using Phaser [17] were not successful. However, when two domains of EcDHDPR were used as independent search models, an MR solution was obtained with a log likelihood gain of 338. The model was further refined using Refmac5 with three TLS groups that were defined based on an analyses with the TLSMD server.

3. Results and discussion

3.1. Crystal structure of SaDHDPR

The crystal structure of SaDHDPR was determined at 1.8 Å resolution-representing the highest resolution structure of a bacterial DHDPR. While SaDHDPR crystallized in several conditions, the resolution of this data was restricted due to an unusually long unit cell axis [19]. Removal of the hexa-histidine tag from the recombinant protein was crucial to obtain crystals that diffracted to high resolution. However, despite extensive efforts, we could not obtain crystals of the SaDHDPR-cofactor complexes. Each monomer unit of SaDHDPR comprises of 240 residues, with an N-terminal nucleotide binding domain and a C-terminal substrate binding domain connected by a hinge region. Discontinuous electron density in the nucleotide and substrate binding cavities were interpreted as sulfate ions as the crystallization condition contained ammonium sulfate. In case of chain B and D, flat trigonal density was modeled as acetate as the crystallization condition also contained sodium acetate. The four monomer units of SaDHDPR interact extensively with an overall buried surface area of 10 890 Å2 (Supplementary Table 1). The tetrameric arrangement of SaDHDPR is consistent with both gel filtration and dynamic light scattering studies (Supplementary Fig. 1).

### Table 1

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<th>Summary of data collection and refinement statistics.</th>
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* Values for outer shells are given in parenthesis.

* a Resolution represents the highest resolution structure of a bacterial DHDPR.
The N-terminal nucleotide binding domain of SaDHDPR comprises residues from 1 to 103 and 214 to 240. As observed in most DHDPR enzymes [10,11,18], this domain has a Rossmann-fold $\alpha/\beta$ structure consisting of four $\alpha$-helices and seven $\beta$-strands (Fig. 1). The C-terminal domain of SaDHDPR comprises residues from 108 to 211 and consists of a mixed $\beta$-sandwich formed by two $\alpha$-helices (a4-a5) and four $\beta$ strands (b7-b10) (Fig. 1a and b). These eight $\beta$-strands from two adjacent DHDPR units interact extensively with the $\beta$-strands of the opposing unit to form a sixteen-stranded central $\beta$-barrel which forms the scaffold for the tetrameric assembly. In addition, a C-terminal long loop connecting the second helix (a5) to second strand (b8) of the substrate binding domain, provides additional strength to the tetrameric association.

Fig. 1. Sequence and structural features of S. aureus DHDPR. (a) Structure based sequence alignment of SaDHDPR (wheat), EcDHDPR (green) TmDHDPR (cyan), MtDHDPR (orange) and BhDHDPR (blue) enzymes (MUSTANG server; [27]). Residues in the nucleotide binding (NTD) and dihydrodipicolinate (DHP) binding motif are indicated by a box. Residues at the DHP-binding motif are highly conserved, while those at the NTD-binding motif are not. In particular, residues equivalent to EcDHDPR Arg39 and Lys9 and Lys11 of MtDHDPR (highlighted in red) are not conserved in SaDHDPR. The residues corresponding to the N-terminal and C-terminal long loop regions are highlighted in black. Additional sequence and structural analysis is presented in Supplementary Figs. 2 and 3. (b) Analysis of sequence conservation of DHDPR enzymes in the context of the SaDHDPR structure. This figure was made using the CONSURF server [28]. (c) Structural comparison of SaDHDPR with homologues. The structures (colors) correspond to SaDHDPR (wheat), EcDHDPR (green), TmDHDPR (cyan) and MtDHDPR (orange) and BhDHDPR (blue). The N-terminal long loop and C-terminal long loops vary in their conformation. The N-terminal long loop is shorter in SaDHDPR (residues Thr33 to Pro39) when compared to EcDHDPR (residues Glu40 to Gly59) and BhDHDPR (residues Lys35 to Gly54), while it is absent in TmDHDPR and MtDHDPR. The C-terminal long loop in SaDHDPR (residues Leu157 to Pro176) is comparable to EcDHDPR (Leu182 to Gly204) and BhDHDPR (Arg177 to Ile201). This loop is absent in TmDHDPR and adopts a different conformation in MtDHDPR.
Despite having similar overall structure and quaternary arrangement, SaDHDPR shows prominent differences from known DHDPR structures [10,11,18]. A loop between β-strands (also known as N-terminal long loop) varies from previously determined DHDPR structures (Fig. 1c). This loop is much shorter in SaDHDPR (residues Thr33 to Pro39) when compared to EcDHDPR (residues Glu40 to Gly59) and B. henselae DHDPR (BhDHDPR) (residues Lys35 to Gly54) ([12]; PDB: 3IJP). The C-terminal long loop in SaDHDPR (residues Leu157 to Pro176) is comparable to that of EcDHDPR (residues Leu182 to Gly204) and BhDHDPR comprising residues Arg177 to Ile201 (Fig. 1c). This loop is absent in Tm DHDPR [14] and adopts a different conformation in MtDHDPR [11].

3.2. Variation in the inter-domain angle of SaDHDPR monomer units

The structures of EcDHDPR and MtDHDPR determined as binary complexes (bound to either NADH or NADPH) and ternary complexes (bound to substrate analogues along with NADH or NADPH) revealed a rotation of the N-terminal domain towards the C-terminal domain upon substrate binding [10,11]. A comparison between the crystal structures of DHDPR homologues suggests that this enzyme can adopt a variety of conformations due to different orientations between the N-terminal and the C-terminal domain [18].

An analysis of different monomer units of SaDHDPR reveals significant differences in the orientation between the two domains. To calculate the inter-domain angle, the angle between centre of mass (COM) of the N-terminal domain (residues 1–103 and 214–240), the hinge segment (Ser107 and Ser212) and the C-terminal domain (residue 108–211) was considered. This angle corresponds to about 124° in case of chain C and D, while it is 117° and 113° in case of chain A and B respectively (Fig. 2a; Supplementary Table 2). A comparison of these orientation angles reveals that chain B of SaDHDPR is closer to the canonical closed conformation, chain A is partially open, whereas chains C and D adopt the open conformation. It is important to note that no electron density was observed in any of the SaDHDPR monomer units to model either the nucleotide cofactor (NADH or NADPH) or the substrate. The different monomer units of SaDHDPR thus not only represent different domain orientations but also belie the generalization that binding of both nucleotides and substrates are essential to trigger domain re-orientation [18]. This observation is significant as studies on the E. coli DHDPR reveal that NADH binding occurs in two steps, with high affinity binding to only one monomer unit. Indeed, both substrate and co-factor binding characteristics have been successfully utilized for the design of bi-ligand inhibitors for this enzyme [20].

The conformationally flexible and rigid regions of SaDHDPR were analyzed using ESCET [21]. This analysis was performed with the standard default parameters in this program. Except for the C-terminal long loop region (residues Glu159 to Gln177), most of the C-terminal domain was defined as largely rigid. The N-terminal domain was conformationally flexible with helix-a2 (Phe58 to Phe72) and helix-a3 (residues Ala79 to Leu94) being more flexible than the rest of the regions (Fig. 2b). In addition, two segments at the inter-domain region, residues Ser107 to Gly109 and Ser212 to Ile215 were also largely flexible. Based on the ESCET analysis, it appears likely that the entire N-terminal domain can spontaneously reorient between the open and closed conformations. This finding is consistent with that reported for MtDHDPR [18].

3.3. Nucleotide specificity of SaDHDPR

The co-factor specificity for EcDHDPR and MtDHDPR is similar—both demonstrate a higher affinity for NADH over NADPH [11,12]. TmDHDPR has a higher affinity for NADPH [14]. An alignment of SaDHDPR with other DHDPR sequences suggests minor variations in the NAD and the DHP binding motifs (Fig. 1a; Supplementary Fig. 2). For example, an acidic residue that is proposed to interact with 3’-OH of bound NADH (equivalent to EcDHDPR Glu38 and Glu39) is

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**Fig. 2.** Domain movements in SaDHDPR. (a) Structural superposition of the closed (chain B; violet) and open (chain D; teal) forms of SaDHDPR. The difference in the orientation of the two domains in the two conformers corresponds to a rotation of about 11°. The centers of mass of each domain in both conformers are depicted as spheres. (b) The Error-inclusive Structure Comparison and Evaluation Tool (ES CET) analysis of four chains of SaDHDPR was carried out to identify conformationally flexible and rigid regions [21]. The lower and upper tolerance limits used were 7ε and 12ε. The C-terminal domain is conformationally invariant (blue), except for the C-terminal long loop. The N-terminal domain as a whole is flexible, with regions in green being moderately flexible while those in red are highly flexible.
MtDHDP R Asp33) is conserved in SaDHDP R (Glu31). However, nei-
ther the equivalent of EcDHDP R Arg39 nor the functionally equiv-
alent Lys9 and Lys11 of MtDHDP R are conserved in SaDHDP R. This
observation was intriguing as these residues are important in
NADPH interactions in the case of the E. coli and M. tuberculosis en-
zymes [10,18]. It thus appeared likely that SaDHDP R may show a
distinct preference for NADH over NADPH. In order to understand
the nucleotide preference of SaDHDP R, nucleotide binding of SaD-
HDPR was monitored using isothermal titration calorimetry. In this
experiment, SaDHDP R (75 μM) in the sample cell was titrated
against either NADH or NADPH (2 mM) in separate experiments
(Fig. 3). It is pertinent to note, in this context, that the ITC

*Fig. 3. Thermodynamic analyses of nucleotide binding to SaDHDP R. Panels (a) and (c) represent NADPH binding profiles of the wild-type and the K35A variant of SaDHDP R
while (b) and (d) represent NADH binding profiles of wild type and the K35A mutant. Each titration was performed at 75 μM protein concentration. The raw data is presented
in the top panel, while the bottom panel shows the fitted titration curve using ORIGIN software.*
experiments were performed at a much higher pH (pH 7.5) when compared to that of the crystallization buffer (pH 4.5). The $K_d$ value of SaDHDPR for NADH is 9.8 $\mu$M and that for NADPH is 0.9 $\mu$M. For comparison, the reported $K_d$ value for EcDHDPR is 0.46 $\mu$M and 2.1 $\mu$M for NADH and NADPH respectively [22].

The co-factor binding data was surprising as it could not be reconciled with either sequence features or surface charge potential differences in SaDHDPR (Fig. 4a and b). While the nucleotide binding pockets of EcDHDPR and SaDHDPR are largely similar, significant differences were seen in the N-terminal loop that connects the second and third strands of the Rossmann fold (b2 and b3). Probably, in order to compensate for the missing cationic residue (Arg39 of EcDHDPR), this loop re-arranges itself such that Lys35 of SaDHDPR occupies a structurally equivalent position. In this hypothesis, Lys35 would interact with the 2’-phosphate of NADPH (Fig. 4c; Supplementary Fig. 3). This was further experimentally validated by the SaDHDPR K35A mutant. The K35A mutation did not alter the oligomeric status or spectroscopic properties of SaDHDPR (Supplementary Fig. 4). However, NADPH binding was altered by ca 20 fold in the K35A mutant enzyme, with much less change (ca 4 fold) in NADH binding (Table 2). The structure of SaDHDPR provides a basis to rationalize this nucleotide preference. Indeed, the role of Lys35 could not have been established from sequence analysis alone as this residue is not conserved across DHDPR homologues (Fig. 1a; Supplementary Fig. 2). On a more general note, the nucleotide preference of SaDHDPR for NADPH over NADH is interesting as many of the pyridine nucleotide dependent enzymes of *S. aureus* including enoyl-acyl carrier protein reductase and coenzyme A-disulfide reductase prefer NADPH over NADH in contrast to their *E. coli* homologues [23,24]. This feature could perhaps be attributed to higher NADPH/NADP$^+$ ratios and low NADH/NAD$^+$ ratios in most gram-positive bacteria [25].

![Fig. 4. Cofactor specificity of SaDHDPR. Panels a and b. The surface charge potential of EcDHDPR (a) and SaDHDPR (b) calculated using APBS [29]. Bound NADPH of EcDHDPR is represented in sticks (green) while the equivalent region of SaDHDPR is highlighted. (c) A comparison of the nucleotide binding pocket of EcDHDPR (green) with the equivalent site of SaDHDPR (wheat). Lys35 of SaDHDPR was identified as the residue equivalent to EcDHDPR Arg39. This residue is likely to be involved in the stabilization of the 2’-PO$_4$ of NADPH.](image-url)
The stoichiometry (n) and enthalpy (AH) and entropy (AS) are not reported as they cannot be reliably estimated due to lack of sigmoidal binding.

While this manuscript was under revision, a paper reporting a similar observation on the co-factor specificity of SaDHDPR was published [26].

4. Conclusion

The structure of S. aureus DHDPR reveals several differences when compared to other DHDPR homologues. The orientation between the co-factor binding and tetramerization domains exemplifies conformational sampling and suggests the extend of domain dynamics in this enzyme even in the absence of bound ligands. Although SaDHDPR lacks a conserved basic residue crucial for NADPH interaction, it exhibits a higher affinity for NADPH over NADH. A comparison of the SaDHDPR structure with EcDHDPR suggested that a non-conserved residue in the N-terminal long loop, Lys35, could perform the equivalent role of EcDHDPR Arg39. The nucleotide binding characteristics of the K35A mutant provides experimental evidence for this hypothesis. Put together, these observations could substantially facilitate on-going programs for bi-ligand inhibitor design that relies on ligand-induced conformational changes as well as co-factor specificity for this important drug target.

The coordinates and structure factors for S. aureus DHDPR have been deposited with the Protein Data Bank (PDB:3QY9).

Appendix A. Supplementary data


References