Soares da Costa et al., 2016, Structure 24, 1282–1291
August 2, 2016 © 2016 Elsevier Ltd.
http://dx.doi.org/10.1016/j.str.2016.05.019

Structural Determinants Defining the Allosteric Inhibition of an Essential Antibiotic Target

Highlights
- Crystal structure of *L. pneumophila* DHDPS and lysine-bound *S. pneumoniae* DHDPS
- DHDPS allosteric inhibition is not defined by Gram staining
- Glu or His at position 56 in DHDPS defines lysine binding
- DHDPS enzymes with Lys or Arg at position 56 are not inhibited by lysine

In Brief
In some bacteria, lysine biosynthesis is regulated by lysine-mediated allosteric inhibition of the enzyme dihydrodipicolinate synthase (DHDPS). Soares da Costa et al. show that position 56 (*E. coli* numbering) defines DHDPS allostery, dispelling the current dogma that regulation is based on Gram staining.
Structural Determinants Defining the Allosteric Inhibition of an Essential Antibiotic Target


INTRODUCTION

Dihydrodipicolinate synthase (DHDPS) catalyzes the first committed step in the lysine biosynthesis pathway of bacteria. The pathway can be regulated by feedback inhibition of DHDPS through the allosteric binding of the end product, lysine. The current dogma states that DHDPS from Gram-negative bacteria are inhibited by lysine but orthologs from Gram-positive species are not. The 1.65-Å resolution structure of the Gram-negative Legionella pneumophila DHDPS and the 1.88-Å resolution structure of the Gram-positive Streptococcus pneumoniae DHDPS bound to lysine, together with comprehensive functional analyses, show that this dogma is incorrect. We subsequently employed our crystallographic data with bioinformatics, mutagenesis, enzyme kinetics, and microscale thermophoresis to reveal that lysine-mediated inhibition is not defined by Gram staining, but by the presence of a His or Glu at position 56 (Escherichia coli numbering). This study has unveiled the molecular determinants defining lysine-mediated allosteric inhibition of bacterial DHDPS.

SUMMARY

Dihydrodipicolinate synthase (DHDPS) catalyzes the first committed step in the lysine biosynthesis pathway of bacteria. The pathway can be regulated by feedback inhibition of DHDPS through the allosteric binding of the end product, lysine. The current dogma states that DHDPS from Gram-negative bacteria are inhibited by lysine but orthologs from Gram-positive species are not. The 1.65-Å resolution structure of the Gram-negative Legionella pneumophila DHDPS and the 1.88-Å resolution structure of the Gram-positive Streptococcus pneumoniae DHDPS bound to lysine, together with comprehensive functional analyses, show that this dogma is incorrect. We subsequently employed our crystallographic data with bioinformatics, mutagenesis, enzyme kinetics, and microscale thermophoresis to reveal that lysine-mediated inhibition is not defined by Gram staining, but by the presence of a His or Glu at position 56 (Escherichia coli numbering). This study has unveiled the molecular determinants defining lysine-mediated allosteric inhibition of bacterial DHDPS.

Dihydrodipicolinate synthase (DHDPS) (EC 4.3.3.7) is an allosteric enzyme that catalyzes the first committed step in the lysine biosynthesis pathway of bacteria and plants (Dogovski et al., 2009, 2012; Soares da Costa et al., 2015) (Figure 1). This pathway produces key building blocks for the synthesis of housekeeping proteins, virulence factors, and the peptidoglycan cell wall in bacteria (Dogovski et al., 2009, 2012; Soares da Costa et al., 2015). It is therefore not surprising that DHDPS is the product of an essential bacterial gene (Becker et al., 2006; Dogovski et al., 2013; Forsyth et al., 2002; Kobayashi et al., 2003). Given its essentiality to pathogenic bacteria and absence in humans, DHDPS is considered a promising antibiotic target (Hutton et al., 2007). This has generated considerable interest in characterizing the structure, function, and inhibition of this bacterial enzyme.

DHDPS exists as dimers or tetramers with the dimeric unit containing all the molecular requirements for catalysis and allosteric inhibition (Dobson et al., 2005b; Mirwaldt et al., 1995). However, tetramers are more commonly observed in both Gram-negative and Gram-positive bacteria, most likely because tetramerization stabilizes conformational dynamics to afford optimal enzymatic function (Griffin et al., 2008, 2010; Voss et al., 2010). By contrast, dimeric forms of DHDPS have evolved to stabilize dynamics by increasing the buried surface area at the dimeric interface (Burgess et al., 2008a).

Functionally, DHDPS catalyzes the condensation of pyruvate and (S)-aspartate semialdehyde (ASA) to form the heterocyclic product, hydroxytetrahydrodipicolinic acid (Figure 1). Not surprisingly, traditional DHDPS inhibition strategies have focused on developing small molecules with analogy to these substrates and/or products (Boughton et al., 2008; Hutton et al., 2003, 2007; Laber et al., 1992; Mitsakos et al., 2008; Turner et al., 2005a, 2005b). The most potent substrate-analog inhibitor discovered to date is dipicolinic acid N-oxide, which has a half-maximal inhibitory concentration (IC_{50}) value of 0.8 mM (Couper et al., 1994). The DHDPS structure also contains a “druggable” allosteric cleft that binds the natural inhibitor, lysine. This mediates a canonical feedback inhibition response as depicted in Figure 1. Lysine is a potent inhibitor of bacterial DHDPS, with IC_{50} values ranging from 53 μM to 1 mM (Bakhiet et al., 1984; Devenish et al., 2009; Joerger et al., 2003; Laber et al., 1992; Skovpen and Palmer, 2013; Soares da Costa et al., 2010; Tam et al., 2004; Yugar and Gilvarg, 1965), providing scope to incorporate rational drug design efforts to afford the discovery of high-affinity...
allosteric inhibitors (Skovpen et al., 2016). In the allosteric binding site, two lysine molecules bind in a bis conformation in close proximity (Cα atoms ~4 Å apart), with the side-chain ε-amino groups projecting away from each other (Dobson et al., 2005b). The bound lysine molecules are stabilized via a number of hydrogen bonding interactions mediated primarily by Ser48, Ala49, Leu51, His53, His56, Asn80, Glu84, and Tyr106 (Escherichia coli DHDPS numbering) (Dobson et al., 2005b). The precise nature of lysine-mediated inhibition is not fully understood, and the same mechanism may not be shared by all lysine-inhibited DHDPS enzymes. Previous studies suggest that lysine binding changes the position of Tyr106 (E. coli numbering), which disrupts the hydrogen bonding network involving the general acid-base Tyr107 (E. coli numbering) and triggers changes in conformational flexibility of substrate-binding active-site residues (Atkinson et al., 2013; Dobson et al., 2005b).

Surprisingly, not all bacterial DHDPS enzymes are allosterically inhibited by lysine (Dogovski et al., 2009, 2012; Soares da Costa et al., 2015), with both dimeric and tetrameric forms allosterically inhibited in some bacterial species but not in others (Burgess et al., 2008a; Dobson et al., 2005a; Kaur et al., 2011; Voss et al., 2010). This demonstrates that oligomerization plays no role in allosteroy. Indeed, the current dogma suggests that DHDPS from Gram-negative bacteria are inhibited by lysine (Bakhiet et al., 1984; Devenish et al., 2009; Dobson et al., 2005b; Joerger et al., 2003; Kaur et al., 2011; Laber et al., 1992; Skovpen and Palmer, 2013; Soares da Costa et al., 2010; Tam et al., 2004b; Yugari and Gilvarg, 1965); whereas the enzymes from Gram-positive bacteria are insensitive to allosteric regulation (Burgess et al., 2008a; Cahyanto et al., 2006; Cremer et al., 1988; Domigan et al., 2009; Halling and Stahly, 1976; Voss et al., 2010; Webster and Lechowich, 1970; Yamakura et al., 1974). The lack of lysine sensitivity in Gram-positive bacteria has been attributed to the high lysine content of their cell walls (Slade and Slamp, 1962).

Here we initially set out to compare the structure and function of DHDPS from two common pneumonia-causing pathogens, namely the Gram-negative bacterium Legionella pneumophila (Lp) and the Gram-positive bacterium, Streptococcus pneumoniae (Sp). Strikingly, we show that SpDHDPS is the first example of a DHDPS from a Gram-positive pathogen that is allosterically inhibited by lysine. Conversely, we demonstrate that the ortholog from L. pneumophila is the first DHDPS from a Gram-negative pathogen that lacks allosteric inhibition. This prompted a re-evaluation of the dogma and the identification of the molecular determinants that define lysine-mediated allosteric inhibition of DHDPS. As proof of concept, a selection of Gram-negative (Burkholderia pseudomallei and Coxiella burnetii) and Gram-positive (Enterobacter faecalis and Campylobacter sp. 17–4) DHDPS enzymes that were predicted to be sensitive and insensitive to lysine inhibition based on the newly identified determinants were examined. Our predictions were confirmed by employing a combination of bioinformatics, mutagenesis, protein biochemistry, biophysics, and enzymology. Thus we are now able to reliably predict the presence or absence of lysine-mediated inhibition of bacterial DHDPS.

### RESULTS

#### Expression, Purification, and Primary Structure Analysis of LpDHDPS and SpDHDPS

The dapA gene encoding DHDPS was amplified from genomic DNA isolated from L. pneumophila and S. pneumoniae and cloned into the pET11a expression vector, and the recombinant DHDPS enzymes were overexpressed in E. coli and purified to >98% homogeneity as described in Experimental Procedures (Tables 1 and 2).

#### Solution Properties of LpDHDPS and SpDHDPS

Having confirmed the identity of recombinant LpDHDPS and SpDHDPS, we assessed the secondary structure of the proteins using circular dichroism (CD) spectroscopy. The CD spectra of the recombinant enzymes show broad double minima spanning 210–225 nm, which is consistent with the (β/α)9 or TIM-barrel topology reported for DHDPS enzymes from different species (Atkinson et al., 2014; Blickling et al., 1997; Burgess et al., 2008b; Dogovski et al., 2013; Griffin et al., 2008, 2012; Mirwaldt et al., 1995; Voss et al., 2010) (Figure S1). To compare the quaternary
structure of LpDHDPS and SpDHDPS in solution, we performed sedimentation velocity studies in the analytical ultracentrifuge. The results show that LpDHDPS ($M_r = 31,581.0$) sediments as a single species with a standardized sedimentation coefficient ($s_{20, w}$) of 4.3 S (Figure S2A). This value compares well with the 4.2 S observed for the *Staphylococcus aureus* (Sa) DHDPS dimer (Burgess et al., 2008a). Conversion of the $c(s)$ profile to a $c(M)$ distribution reveals an apparent molar mass of 61.4 kDa, consistent with the theoretical mass of an LpDHDPS dimer. In comparison, SpDHDPS ($M_r = 33,780.54$) sediments as 7.4 S with a molecular mass of 131 kDa, consistent with a tetramer as previously described (Dogovski et al., 2013) (Figure S2B). We were therefore interested in comparing the catalytic function of the LpDHDPS dimer and SpDHDPS tetramer.

**Functional Comparison of LpDHDPS and SpDHDPS**

Enzyme kinetics were performed in the absence and presence of (S)-lysine using the quantitative DHDPS-DHDPR coupled assay (Dobson et al., 2005b). Regarding SpDHDPS, the resulting kinetic analysis of LpDHDPS in the absence of lysine demonstrates that the enzyme catalyzes the condensation of (S)-ASA and pyruvate via a typical $bibi$ ping-pong mechanism with no substrate inhibition (Table 3). However, in the presence of increasing concentrations of (S)-lysine, LpDHDPS surprisingly maintains 100% catalytic activity even up to the non-physiological concentration of 5 mM (Figure 2A). This is the first DHDPS enzyme from a Gram-negative pathogen to show a lack of lysine feedback inhibition. By contrast, the Gram-negative control, *E. coli* (Ec) DHDPS, is inhibited by lysine with an $IC_{50}$ of 0.18 mM ($R^2 = 0.988$), agreeing with previously published data (Soares da Costa et al., 2010) (Figure 2A). Even more striking is the observation that SpDHDPS is inhibited by lysine, which is the first case of lysine feedback inhibition reported for a Gram-positive bacterium (Table 3, Figure 2B). For comparison, the activity of DHDPS from the Gram-positive control, *S. aureus*, shows no dose response (Figure 2B). Subsequent in-depth kinetic analyses showed that the mechanism of inhibition for SpDHDPS is mixed with respect to both substrates, resulting in inhibition constants ($K_i$) for (S)-ASA of $K_{i_{ASA}} = 0.031$ mM and $K_{i_{ASA}} = 0.026$ mM; and for pyruvate of $K_{i_{PYR}} = 0.054$ mM and $K_{i_{PYR}} = 0.012$ mM (Figure S3). This demonstrates that the inhibition of SpDHDPS by lysine is at least 3-fold more potent compared with EcDHDPS (Ghislain et al., 1990; Karsten, 1997; Soares da Costa et al., 2010). Given these surprising results, we were interested in determining and comparing the high-resolution crystal structures of apo (i.e., unliganded) LpDHDPS and SpDHDPS bound to lysine.

**Crystal Structure Determination**

To provide insight into the structural determinants that define the presence or absence of lysine-mediated allosteric inhibition of DHDPS, we determined the crystal structures of apo LpDHDPS (PDB: 4NQ1) and lysine-bound SpDHDPS (PDB: 4FH4) to 1.65-Å and 1.88-Å resolution, respectively, using the molecular replacement method. The data collection, processing, scaling, and refinement statistics are presented in Table 4. The overall sequence identity between the two orthologs is 36% and the root-mean-square deviation (rmsd) over 290 residues between the structures is 1.6 Å.

Consistent with our analytical ultracentrifuge data showing that LpDHDPS exists as a dimer in solution, two molecules of LpDHDPS are observed in the asymmetric unit (Figure 3A), as the canonical DHDPS dimer. The extensive interface comprises a total of 17 hydrogen bonds and a buried interface area of 1,418 Å². Comparison of the LpDHDPS and EcDHDPS demonstrates a high degree of conservation in the spatial orientation of almost all active-site residues (Figure S4). The exception is Tyr106, which shows ~90° rotation relative to its *E. coli* counterpart, also observed for the dimeric SaDHDPS (Burgess et al., 2008a; Girish et al., 2008) (PDB: 3DAQ and 3DI0). Shown in Figure 3B is an overlay of the *L. pneumophila* (green) and *E. coli* (purple) DHDPS allosteric sites. Depicted in orange is a lysine ligand bound to the *E. coli* site (PDB: 1YXD), which makes key contacts with His56, Tyr106, and Glu84. In LpDHDPS (PDB: 4NQ1) these residues are replaced with Lys55, Ala105, and Asp83, respectively. The side chain of Lys55 lies in close proximity to the ε-amino moiety of the bound lysine in the *E. coli* enzyme. This introduces potential electrostatic repulsion and steric hindrance that would prevent lysine binding into the LpDHDPS cleft. Furthermore, the carboxyl group of the lysine molecule is coordinated by the phenolic hydroxyl group of Tyr106 in EcDHDPS. A substitution to alanine eliminates this stabilizing interaction. Finally, Glu84 in EcDHDPS participates in a hydrogen bonding network with Ala49 and Asn80 to secure the

---

**Table 2. Electrospray Ionization Time-of-Flight Mass Spectrometry Results of Purified Recombinant DHDPS**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Predicted Molecular Mass (Da)</th>
<th>Experimental Molecular Mass (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. pneumophila</em></td>
<td>31,581.0</td>
<td>31,580.4</td>
</tr>
<tr>
<td><em>S. pneumoniae</em></td>
<td>33,780.5</td>
<td>33,780.2</td>
</tr>
<tr>
<td><em>B. pseudomallei</em></td>
<td>31,665.0</td>
<td>31,664.7</td>
</tr>
<tr>
<td><em>E. faecalis</em></td>
<td>35,659.8</td>
<td>35,659.8</td>
</tr>
<tr>
<td><em>C. burnetii</em></td>
<td>35,742.9</td>
<td>35,740.9</td>
</tr>
<tr>
<td><em>Campylobacter sp. 17-4</em></td>
<td>35,972.9</td>
<td>35,973.6</td>
</tr>
<tr>
<td><em>E. faecalis E56K</em></td>
<td>34,942.1</td>
<td>34,941.2</td>
</tr>
</tbody>
</table>

The deconvoluted mass spectra reveal that the predominant peaks are consistent with the theoretical molecular mass for each protein.

---

**Table 3. Kinetic Constants for LpDHDPS and SpDHDPS**

<table>
<thead>
<tr>
<th>Protein</th>
<th>$K_{cat}$ (s$^{-1}$)</th>
<th>$K_M^{ASA}$ (mM)</th>
<th>$K_M^{PYR}$ (mM)</th>
<th>IC$_{50}$ Lysine (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LpDHDPS</td>
<td>101</td>
<td>0.241 ± 0.013</td>
<td>0.186 ± 0.010</td>
<td>no inhibition</td>
</tr>
<tr>
<td>SpDHDPS</td>
<td>22.2*</td>
<td>0.0442 ± 0.0028</td>
<td>0.630 ± 0.006</td>
<td></td>
</tr>
</tbody>
</table>

See also Figure S3.

*Kinetic properties from Dogovski et al. (2013).*
α-amino of the inhibitory lysine. An overlay of Asp83 and Glu84 reveals a significant rotation, as well as displacement, in their respective side chains (Figure 3B). Importantly, the side chain of Asp83 is oriented so that it cannot interact with either bound lysine or other allosteric site residues.

The crystal structure of SpDHDPS in the presence of lysine (PDB: 4FHA) was next assessed. The lysine-bound enzyme forms a tetramer (Figure 4A), consistent with the apoenzyme (Dogovski et al., 2013), with one lysine bound per subunit (Figure 4B). Similar to EcDHDPS, two molecules of lysine are observed bound in a bis conformation in the allosteric cleft formed at the dimer interface adjacent to the active site. However, unlike EcDHDPS, substantial conformational changes are observed when comparing the apo and lysine-bound structures, including significant rotations of His59 (His53, E. coli numbering) and Asp90 (Glu84, E. coli numbering). This results in significant movement, or “tensing,” of the two monomers closer together at the dimer interface, as well as an increase in the number of non-covalent interface contacts (Figure 4B). This may serve to decrease the accessibility of the substrates to the active site and/or the products from exiting. In addition, an electrostatic contact between bound lysine and Glu62 is observed in the SpDHDPS structure (Figure 4B), which is absent in the lysine-bound structure of EcDHDPS. Thus, the presence of this interaction may contribute to the tighter binding of lysine observed for SpDHDPS, consequently inhibiting catalysis.

Identification of the DHDPS Molecular Determinants Defining Lysine Binding

Given our surprising results demonstrating for the first time that lysine allosteric inhibition is not related to Gram staining as previously thought, we set out to characterize the structural determinants that define DHDPS-lysine interactions. Structural analyses comparing the sequence and chemical composition of allosteric site residues from DHDPS enzymes shown to be inhibited by lysine (e.g., PDB: 3M5V, 1YXD, 3FLU, and 4FHA) allowed the mapping of key residues that form interactions with the allosteric ligand. In E. coli (PDB: 1YXD), 16 residues (eight from each subunit) surround the two lysine molecules bound in the allosteric cleft, namely Ser48, Ala49, Leu51, His53, His56, Asn80, Glu84, and Tyr106. These residues form a series of hydrogen bonds either directly with the bound ligand or via coupled networks with the side-chain or backbone moieties of nearby residues. When compared with allosteric DHDPS enzymes from other Gram-negative species, it is noted that the ortholog from Campylobacter jejuni (PDB: 3M5V) has an allosteric site identical to that of the E. coli DHDPS, whereas the enzyme from Neisseria meningitidis (PDB: 3FLU) differs only at a single position, with Val replacing His53 (Figure S5). By contrast, there are some notable differences in the allosteric site of DHDPS from the Gram-positive lysine-sensitive SpDHDPS, with Pro, Glu, and Asp replacing Ala49, His56, and Glu84. Despite these differences, a similar hydrogen bonding network is formed between the allosteric site residues and the ligand in S. pneumoniae and E. coli DHDPS enzymes. Interestingly, comparison of DHDPS protein sequences from species known to be insensitive to lysine allostery (Bacillus anthracis, Clostridium botulinum, L. pneumophila, and S. aureus) shows good conservation in the allosteric cleft, except for position 56 (E. coli numbering), which is a basic (i.e., Lys) residue (Figure S5). We therefore hypothesize that DHDPS sequences containing His or Glu at position 56 will be allosterically inhibited by lysine, whereas those containing a basic (i.e., Lys or Arg) residue at this position will be insensitive to lysine-mediated allostery.

Testing the Newly Identified Determinant of Lysine Binding

A multiple sequence alignment of uncharacterized DHDPS sequences from the Gram-negative intracellular pathogen B. pseudomallei and the Gram-positive bacterium E. faecalis shows that these sequences contain a histidine and a glutamate at position 56, respectively, suggesting that these enzymes will be sensitive to lysine-mediated allostery (Figure 5). In comparison, the DHDPS sequences from the Gram-negative intracellular...
Table 4. Data Collection, Processing, and Refinement Statistics for LpDHDPS and SpDHDPS Co-crystallized with (S)-lysine

<table>
<thead>
<tr>
<th></th>
<th>LpDHDPS (PDB: 4NQ1)</th>
<th>Lysine-Bound SpDHDPS (PDB: 4FHA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wavelength (Å)</td>
<td>0.9357</td>
<td>0.9357</td>
</tr>
<tr>
<td>No. of images</td>
<td>129</td>
<td>720</td>
</tr>
<tr>
<td>Oscillations (°)</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Space group</td>
<td>P6 22</td>
<td>P2 1 2</td>
</tr>
<tr>
<td>Unit cell parameters (Å)</td>
<td>a = 89.31,  a = 106.37, b = 89.31, b = 106.23, c = 290.18, c = 60.38</td>
<td></td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>36.27–1.65 (1.74–1.65)</td>
<td>39.91–1.88 (1.92–1.88)</td>
</tr>
<tr>
<td>Observed reflections</td>
<td>376,126 (58,890)</td>
<td>772,032 (12,445)</td>
</tr>
<tr>
<td>Unique reflections</td>
<td>81,908 (11,872)</td>
<td>55,372 (2,700)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>98.9 (99.8)</td>
<td>98.0 (77.0)</td>
</tr>
<tr>
<td>Rmerge (%)</td>
<td>0.108 (0.343)</td>
<td>0.094 (0.481)</td>
</tr>
<tr>
<td>Rmerge (b)</td>
<td>0.055 (0.169)</td>
<td>0.026 (0.210)</td>
</tr>
<tr>
<td>Mean I/σ(I)</td>
<td>9.1 (4.2)</td>
<td>20.6 (1.90)</td>
</tr>
<tr>
<td>Multiplicity</td>
<td>4.6 (5.0)</td>
<td>13.9 (4.6)</td>
</tr>
<tr>
<td>Wilson B value (Å2)</td>
<td>11.8</td>
<td>21.7</td>
</tr>
<tr>
<td>Molecules per asymmetric unit</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Vm (Å³ Da⁻¹)</td>
<td>2.64</td>
<td>2.55</td>
</tr>
<tr>
<td>Solvent content (%)</td>
<td>53.52</td>
<td>51.84</td>
</tr>
<tr>
<td>Rcryst/Rfree (%)</td>
<td>14.7/18.9</td>
<td>15.9/18.3</td>
</tr>
<tr>
<td>No. of atoms</td>
<td>918</td>
<td>328</td>
</tr>
<tr>
<td>Protein</td>
<td>4,491</td>
<td>4,757</td>
</tr>
<tr>
<td>Water</td>
<td>918</td>
<td>328</td>
</tr>
<tr>
<td>Rmsd</td>
<td>0.027</td>
<td>0.0073</td>
</tr>
<tr>
<td>Bond angles (°)</td>
<td>2.12</td>
<td>1.085</td>
</tr>
<tr>
<td>Average B factors (Å²)</td>
<td>16.2</td>
<td>20.0</td>
</tr>
<tr>
<td>Protein overall</td>
<td>14.4</td>
<td>20.4</td>
</tr>
<tr>
<td>Main chain</td>
<td>15.7</td>
<td>21.1</td>
</tr>
<tr>
<td>Side chain</td>
<td>31.5</td>
<td>23.0</td>
</tr>
<tr>
<td>Solvent</td>
<td>15.9</td>
<td>20.2</td>
</tr>
<tr>
<td>Ligands</td>
<td>92.4</td>
<td>90.8</td>
</tr>
<tr>
<td>Disallowed region</td>
<td>0.4</td>
<td>0.8</td>
</tr>
</tbody>
</table>

Validation of the Lysine Binding Determinants

To confirm that the nature of the residue at position 56 dictates lysine binding and inhibition, we mutated the naturally occurring glutamate at position 56 in EdFHDPS to a lysine (EdFHDPS-E56K). The mutant enzyme was expressed and purified as described for the wild-type enzyme (see Experimental Procedures). Lysine binding was then compared for the wild-type and mutant enzymes using microscale thermophoresis (Wienken et al., 2010). The Kᵦ for EdFHDPS was determined to be 148 ± 10 μM, which agrees well with the IC₅₀ value of 172 μM reported earlier. On the other hand, the mutant enzyme was unable to bind lysine even at 10 mM concentration (Figure 7A). The thermodynamic results for the mutant were confirmed kinetically using the coupled assay, indicating that, although catalytically active, EdFHDPS-E56K had become insensitive to lysine-mediated inhibition (Figure 7B).

DISCUSSION

We show in this study that DHDPS from the Gram-negative pathogen *L. pneumophila* and the Gram-positive bacterium *S. pneumoniae* do not conform to the current dogma for lysine inhibition. The underlying molecular basis for the lack of allostery in *L. pneumophila* was investigated by determining the crystal structure of the enzyme. Inspection of the allosteric pocket confirmed the absence of three key residues; His56, Tyr106, and Glu84 (*E. coli* numbering). Lysine-bound structures in the PDB have demonstrated the importance of these residues in binding and stabilizing lysine within the allosteric cleft. An overlay of the DHDPS allosteric sites in *L. pneumophila* and *E. coli* showed that residues involved in interacting with lysine were replaced with those that would either hinder the binding or fail to form the necessary contacts. Moreover, there are no compensating residues, and the putative allosteric site in LpDHDPS is significantly more open than the equivalent site in orthologs that bind lysine.

On the other hand, SpDHDPS is the first DHDPS from a Gram-positive bacterium to be shown to be inhibited by the end product of the lysine biosynthetic pathway. Furthermore, quantitative inhibition assays demonstrate that SpDHDPS exhibits a high...
degree of sensitivity to the inhibitory effects of (S)-lysine when compared with EcDHDPS (Dobson et al., 2004). The structure of SpDHDPS co-crystallized with (S)-lysine reveals a marked conformational change including a large shift in the orientation of His59 (SpDHDPS numbering). These conformational changes are not evident for EcDHDPS in the presence of (S)-lysine compared with the apo structure. The additional electrostatic contact between (S)-lysine and Glu62 observed in the (S)-lysine-bound SpDHDPS structure may contribute to the tighter binding.

In this study, a key position that defines whether a bacterial DHDPS will allosterically bind lysine was identified. A multiple DHDPS sequence alignment highlights a distinction in the nature of the residue at position 56 that we propose is the major determinant for lysine inhibition of DHDPS. We predict that the presence of a histidine or glutamate at position 56 imbues DHDPS with the ability to allosterically bind lysine. These results in no inhibition. Characterization of BpDHDPS, CbDHDPS, CsDHDPS, and EfDHDPS in terms of lysine binding confirmed our hypothesis proposed here. In addition, mutation of the glutamate at position 56 in EfDHDPS to a lysine completely abolished allosteric inhibition as assessed kinetically using the coupled assay and thermodynamically employing microscale thermophoresis, further validating our findings. We have therefore identified and validated a key position that defines allosteric inhibition in DHDPS from different bacterial species, providing insights into the molecular evolution of enzyme allostery. The findings in this study also provide insight into the targeted design of allosteric inhibitors of DHDPS. However, the reason why some DHDPS enzymes have evolved to be allosterically inhibited by lysine remains to be understood. It may be that a bacterium’s environment and life cycle dictate the propensity for regulation by lysine. Regulation at the gene level may also provide sufficient control of this enzyme such that inhibition at the allosteric level is unnecessary. This remains to be explored in future studies.

EXPERIMENTAL PROCEDURES

Cloning, Expression, and Purification of DHDPS

Genomic DNA from L. pneumophila (strain Alcoy) and a clinical isolate of S. pneumoniae (serotype 3) were used in this study. The procedures for amplifying and cloning the dapA gene (encoding DHDPS) from L. pneumophila and S. pneumoniae and for the overexpression and purification of recombinant DHDPS were performed as described previously (Atkinson et al., 2011; Burgess et al., 2008a; Dogovski et al., 2013; Sibarani et al., 2010; Siddiqui et al., 2013). Briefly, dapA encoding DHDPS was PCR-amplified from genomic DNA and cloned into pET11a, pET28a, or pRSET A expression vector (Table 1). The EfDHDPS-E56K mutant was commercially synthesized by Bioneer and subcloned into the expression vector pET28a. Recombinant protein was produced in E. coli BL21-DE3 cells upon induction with 1 mM isopropyl β-D-thiogalactopyranoside in Luria broth at 37°C or 16°C (L. pneumophila DHDPS). Cells were harvested by centrifugation and sonicated in 20 mM Tris (pH 8.0) containing 150 mM NaCl.

Circular Dichroism Spectroscopy

CD spectroscopy was performed using 1-mm quartz cuvettes in the Aviv Model 420 CD spectrometer as previously described (Atkinson et al., 2014; Burgess et al., 2008a; Dogovski et al., 2013; Griffin et al., 2008, 2012; Voss et al., 2010). In brief, wavelength scans were conducted between 200 and 240 nm in 0.5-nm increments with 2-s averaging time at 20°C. Protein samples were prepared in 20 mM Tris and 150 mM NaCl (pH 8.0) at 150 μg ml⁻¹.

Figure 3. Crystal Structure of LpDHDPS

(A) The dimer of LpDHDPS (PDB: 4NQ1) with monomers shown in green and yellow. Note that application of two-fold crystallographic symmetry assemblies a tetramer.

(B) Comparison of DHDPS allosteric sites by overlaying LpDHDPS (green) with E. coli lysine-bound DHDPS (purple, PDB: 1YXD). Lysine is depicted in orange and residues shown are in E. coli numbering.

See also Figures S1, S2, and S4.

Figure 4. Crystal Structure of SpDHDPS

(A) The tetramer of SpDHDPS (PDB: 4FHA) with each monomer depicted in a different color. The solid line shows the dimer interface and the tetramer interface is delineated by a dashed line. (B) Comparison of DHDPS allosteric sites by overlaying one molecule of apo SpDHDPS (green, PDB: 3VFL) with that of lysine-bound SpDHDPS structure (blue, PDB: 4FHA). Oxygen and nitrogen atoms are shown in red and blue, respectively, lysine is shown in orange, and the blue mesh represents the electron density map (mFo - nFc) for lysine contoured at 2.0σ. The overall B factor of lysine is 22.3 Å² with 100% occupancy. Conformational changes are observed for residues His59 (~90° rotation) and Asp90 (~45° rotation) upon binding of (S)-lysine. Residues shown are in S. pneumoniae numbering.

See also Figures S1 and S2.
Presence of (S)-Lysine and Gram-Negative DHDPS Enzymes in the Presence of (S)-Lysine
(A) o-Aminobenzaldehyde assay showing the activity of various DHDPS (5 μM) in the absence and presence of 5 mM (S)-lysine. Purple color indicates DHDPS activity. Well A1, no enzyme control in the absence of lysine; well A2, no enzyme control in the presence of lysine; well A3, no substrate control in the absence of lysine; well A4, no substrate control in the presence of lysine; well B1, EcDHDPS in the absence of lysine; well B2, EcDHDPS in the presence of lysine; well B3, SdDHDPS in the absence of lysine; well B4, SdDHDPS in the presence of lysine; well C1, LpDHDPS in the absence of lysine; well C2, LpDHDPS in the presence of lysine; well C3, SpDHDPS in the absence of lysine; well C4, SpDHDPS in the presence of lysine; well D1, CbDHDPS in the absence of lysine; well D2, CbDHDPS in the presence of lysine; well E1, CbDHDPS in the absence of lysine; well E2, CbDHDPS in the presence of lysine; well E3, EfDHDPS in the absence of lysine; well E4, EfDHDPS in the presence of lysine.

(B) Lysine inhibition affinities (IC₅₀) of DHDPS from newly characterized Gram-negative and Gram-positive bacteria.
to 1.22 m yielding a final DHDPS concentration of 3 m using iMOSFLM (Battye et al., 2011) scaling using SCALA (Evans, 2006). The collection. Indexing and integration of the diffraction data were performed previously described (Siddiqui et al., 2013). Crystals were soaked in reservoir solution containing 20% (v/v) 2-methyl-2,4-pentanediol (MPD) prior to data collection. Thermophoresis was measured at 30°C applying samples to Monolith NT Standard Treated Capillaries (NanoTemper Technologies). Thermophoresis was measured at 30°C with laser off/on/off times of 5 s/30 s/5 s. Experiments were conducted at 20% LED power and 40% MST IR laser power. Data from three independently performed experiments were analyzed (NT.Analysis software version 1.5.41, NanoTemper Technologies) using the signal from Thermophoresis + T-jump.

**Microscale Thermophoresis**

Affinity measurements using microscale thermophoresis (MST) were carried out with a Monolith NT.LabelFree instrument (NanoTemper Technologies). Lysine diluted in water (20 mM to 2.44 μM) was mixed 1:1 with the enzyme, yielding a final DHDS concentration of 3 μM and a dilution series of 10 mM to 1.22 μM for lysine. All experiments were incubated for 30 min at 30°C, before applying samples to Monolith NT Standard Treated Capillaries (NanoTemper Technologies). Thermophoresis was measured at 30°C with laser off/on/off times of 5 s/30 s/5 s. Experiments were conducted at 20% LED power and 40% MST IR laser power. Data from three independently performed experiments were analyzed (NT.Analysis software version 1.5.41, NanoTemper Technologies) using the signal from Thermophoresis + T-Jump.

**Cryocrystallization, Structure Determination, and Refinement**

X-Ray diffraction experiments were carried out at the Australian Synchrotron on the MX1 beamline (Cowieson et al., 2015) using Blu-Ice (McPhillips et al., 2002). LpDHDPS was crystallized using the hanging-drop vapor diffusion method as previously described (Sidiqui et al., 2013). Crystals were soaked in reservoir solution containing 20% (v/v) 2-methyl-2,4-pentanediol (MPD) prior to data collection. Indexing and integration of the diffraction data were performed using IMOSFLM (Battye et al., 2011) scaling using SCALA (Evans, 2006). The crystal was found to belong to space group P61 22. The Matthews coefficient of 2.64 Å3 Da−1 with an estimated solvent content of 53.5% indicated that there were two monomers in the asymmetric unit. Molecular replacement was performed using PHASER (McCoy et al., 2007) with a single chain of Pseudomonas aeruginosa DHDS as the search model (PDB: 3QZE; 54% sequence identity). Iterative model building of the structure and refinement was performed in WINCOOT (Emsley and Cowtan, 2004) and REFMAC5 (Murshudov et al., 1997; Winn et al., 2011), respectively. A lysine bonded to pyruvate as a Schiff base was built in sketcher. Babinet scaling in REFMAC5 was applied in the final rounds of refinement. Structure quality was assessed by MolProbity and WINCOOT (Chen et al., 2010; Davis et al., 2007).

SpDHDPS was co-crystallized with (S)-lysine using the hanging-drop vapor diffusion method as previously described (Dogovski et al., 2013; Sibarani et al., 2013), with (S)-lysine added to the protein solution in a 1:1 ratio (1 μl of concentrated (S)-lysine at 1 M added to 1 μl of concentrated protein solution at 8 mg ml−1). Crystals were soaked in reservoir solution containing 0.2 M sodium fluoride, 20% (w/v) polyethylene glycol 3350, 0.1 M bis-Tris propane (pH 6.0), and 15% (w/v) glycerol prior to data collection at 100 K. A 97.8% complete dataset was integrated to 1.88-Å resolution using XDS (Kabsch, 2010) and scaled using SCALA (Evans, 2006). Data analysis using phenix.xtriage (Zwart et al., 2005) revealed the presence of pseudo-merohedral twinning, which resulted in a space-group change from P412121 to P212121 with a = 106.37 Å, b = 106.23 Å, and c = 60.38 Å, similar to the parent unliganded structure of SpDHDPS (Dogovski et al., 2013). Molecular replacement was carried out using the program PHASER (McCoy et al., 2007) with the structure of the native form of SpDHDPS (PDB: 3FL) using the most complete monomer as the search model, which packed one molecule in the asymmetric unit. The structure was refined using REFMAC5 (Murshudov et al., 1997; Winn et al., 2011), including TLS refinement (Winn et al., 2003). Iterative model building was carried out using the program Coot (Emsley and Cowtan, 2004).

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes five figures and can be found with this article online at http://dx.doi.org/10.1016/j.str.2016.05.019.

**AUTHOR CONTRIBUTIONS**


**ACKNOWLEDGMENTS**

We would firstly like to acknowledge the support and assistance of the friendly staff at the CSIRO Collaborative Crystallisation Center (www.csiro.au/C3), Melbourne, Australia, and the beamline scientists at the Australian Synchrotron, VIC, Australia. We would also like to thank Prof. Elizabeth Hartland (Department of Microbiology and Immunology, The University of Melbourne) for providing genomic DNA from *L. pneumophila* and A/Prof. Geoffrey Hogg (Microbiological Diagnostic Unit, University of Melbourne, Public Health Laboratory Network, Department of Health and Aging, Australia) for providing genomic DNA from *S. pneumoniae*. M.A.P. and S.P. acknowledge the Australian Research Council for funding support (DP150103313), T.P.S.C. and M.W.P. the National Health and Medical Research Council of Australia for fellowship support (APP1091976 and APP1021645). J.J.P. was funded by an
Australian Synchrotron fellowship. Funding from the Victorian Government Operational Infrastructure Support Scheme to St Vincent’s Institute is acknowledged. We would also like to acknowledge the La Trobe University-Comprehensive Proteomics Platform for providing infrastructure and expertise. Finally, we thank all members of the Perugini laboratory for helpful discussions during the preparation of the manuscript.

Received: January 6, 2016
Revised: April 22, 2016
Accepted: May 6, 2016
Published: July 14, 2016

REFERENCES


