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Dihydrodipicolinate Synthase from *Campylobacter jejuni*: Kinetic Mechanism of Cooperative Allosteric Inhibition and Inhibitor-Induced Substrate Cooperativity

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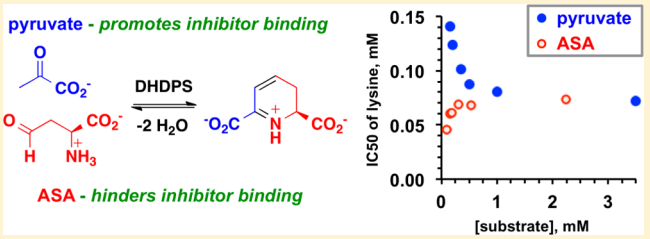
Supporting Information

ABSTRACT: Dihydrodipicolinate synthase (DHDPS), an enzyme of the *meso*-diaminopimelate pathway of lysine biosynthesis, is essential for bacterial growth and is considered a target for novel antibiotics. We have studied DHDPS from *Campylobacter jejuni* for the first time, determining the kinetic mechanism of catalysis and inhibition with its natural allosteric feedback inhibitor (*S*)-lysine. The tetrameric enzyme is known to have two allosteric sites, each of which binds two molecules of lysine. The results suggest that lysine binds highly cooperatively, and primarily to the F form of the enzyme during the ping−pong mechanism. By applying graphical methods and nonlinear regression, we have discriminated between the possible kinetic models and determined the kinetic and inhibition constants and Hill coefficients. We conclude that (*S*)-lysine is an uncompetitive partial inhibitor with respect to its first substrate, pyruvate, and a mixed partial inhibitor with respect to its second substrate, (*S*)-aspartate-*β*-semialdehyde (ASA), which differs from the kinetic models for inhibition reported for DHDPS from other sources. The Hill coefficients for the binding of lysine to different forms of the enzyme are all greater than 2, suggesting that the two allosteric sites are not independent. It has been found that ASA binds cooperatively in the presence of (*S*)-lysine, and the cooperativity of binding increases at near-K_M concentrations of pyruvate. The incorporation of Hill coefficients into the kinetic equations was crucial for determining the kinetic model for this enzyme.



Dihydrodipicolinate synthase (DHDPS, E.C. 4.2.1.52) is an allosterically regulated enzyme of the bacterial *meso*-diaminopimelate pathway, responsible for condensation of (*S*)-aspartate-*β*-semialdehyde (ASA) and pyruvate into an unstable heterocyclic product (4*S*)-hydroxy-2,3,4,5-tetrahydro-(2*S*)-dipicolinic acid,[1,2] which spontaneously dehydrates to (*S*)-2,3-dihydropyridine-2,6-dicarboxylate (dihydrodipicolinate)[2−4] (Figure 1). The reaction has been shown to follow a ping−pong, or "substituted-enzyme" mechanism, whereby pyruvate condenses with an active site lysine residue of the native or "E" form of the enzyme. This substituted or "F" form binds ASA, and a new carbon−carbon bond is formed via an aldol reaction. The ligated intermediate then cyclizes by transimination and is released from the active site.

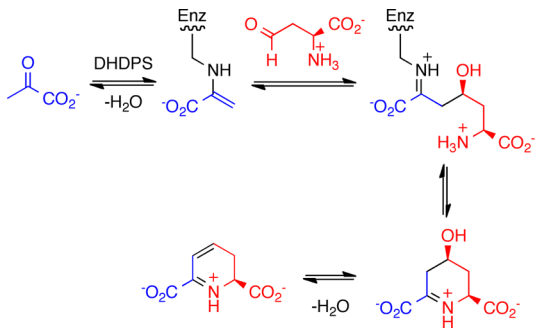


Figure 1. Reaction catalyzed by DHDPS. In the case of *C. jejuni* DHDPS, the active-site lysine residue is Lys166.

DHDPS is an attractive antibiotic target because (*S*)-lysine and *meso*-diaminopimelic acid are cross-linking components between peptidoglycan heteropolysaccharide chains in bacterial cell walls.[5,6] The activity of DHDPS can be affected by inhibitor molecules binding at the active or the allosteric site of the enzyme. A number of compounds targeting both active and allosteric sites have been proposed and described in the literature,[7−13] but effective inhibitors of DHDPS have not yet been reported. In order to design an effective allosteric modulator of DHDPS, it is necessary to clarify the mechanism of allosteric interaction of DHDPS with its natural allosteric inhibitor (*S*)-lysine. There are several reports describing lysine inhibition of DHDPS isolated from different species, such as plants[12,14,15] and Gram-negative bacteria,[16,17] which are inhibited at very low concentrations of lysine, and Gram-positive bacteria,[18−20] which are weakly inhibited or insensitive to lysine. Despite this level of scrutiny, the kinetic mechanism of lysine inhibition is still not clear. Early papers using the wheat enzyme reported that lysine is a competitive inhibitor

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with respect to ASA and a noncompetitive inhibitor with respect to pyruvate.[12] On the contrary, Frisch et al. reported that lysine is a competitive inhibitor versus pyruvate and mixed inhibitor with respect to ASA using the DHDPS from maize.[15] Laber et al. described the mechanism of lysine inhibition of *Escherichia coli* DHDPS as partial,[13] which is in agreement with subsequent reports. Karsten,[21] also working with the *E. coli* enzyme, determined that lysine binds to the F form of the enzyme at pH 8, suggesting an uncompetitive partial mechanism with respect to pyruvate. In the same article, he showed that lysine can bind to the E form of the enzyme at low pH, which is an indication of a mixed model of inhibition. Over the past decade, research groups, particularly Gerrard and coworkers, have been describing the lysine inhibition mechanism as "partial mixed" with respect to pyruvate and "noncompetitive partial" with respect to ASA,[17] except in the report of Soares da Costa et al. that defines the inhibition type as "partial uncompetitive" with respect to pyruvate.[22] Atkinson et al.[23] also reports "mixed partial" and "noncompetitive partial" inhibition with respect to pyruvate and ASA respectively for the common grapevine DHDPS.

With the aid of isothermal titration calorimetry, it was shown that lysine binds cooperatively displaying a cooperative partial mixed model with respect to pyruvate for *E. coli* DHDPS,[24] and lysine was also observed to bind to the E form of *S. meliloti* DHDPS.[25] Importantly, the cooperative mode of lysine binding has been known as early as 1987,[12] but only a few authors reported cooperativity coefficient values,[12,14,21,23] and the cooperativity of binding is not accounted for in kinetic analyses. This may account for the variation in interpretation of kinetic data for DHDPS; although the determination of Hill coefficients and their incorporation into kinetic analyses makes the analysis more complex, it is likely an essential component for determining the best kinetic model of lysine inhibition. (As this manuscript was being prepared, Atkinson et al.[23] incorporated cooperativity coefficients for lysine binding derived from a nonlinear fit to kinetic models.)

Here we report a kinetic analysis of the catalyzed reaction and its allosteric inhibition for the recombinant hexahistidine-tagged DHDPS from *Campylobacter jejuni*. *C. jejuni* is a Gram-negative pathogen that can cause gastroenteritis in humans and can also lead to severe conditions such as Guillain-Barré syndrome, meningitis, and chronic colitis.[26,27] Sequence alignment[28,29] reveals that *C. jejuni* DHDPS shares 37% sequence identity with *E. coli* DHDPS. Kinetic parameters and lysine inhibition constants of *C. jejuni* DHDPS have not yet been reported, but X-ray crystal structures of *C. jejuni* DHDPS bearing an N-terminal hexahistidine tag in the E form (RCSB Protein Data Bank ID 3M5V) and in the pyruvate-modified F form (PDB ID 3LER) have been deposited. These structures show conservation of the tertiary and quaternary structure: a $(\beta/\alpha)_8$ "TIM" barrel monomer forms a tetramer consisting of a loosely associated dimer of more intimately connected dimers. Each "tight dimer" contains an allosteric regulatory site at the interface of the two monomers, and within these dimers each monomer contributes a tyrosine residue to the active site of the other. The presence of the hexahistidine tag appears to have no effect on the tertiary or quaternary structure of the enzyme. All active-site residues identified in DHDPS from other species are present in the *C. jejuni* DHDPS structure, as are the residues responsible for lysine binding at the allosteric site[30] which have been determined as a result of the crystal structure of *E. coli* DHDPS with bound lysine molecules (PDB ID 1YXD). As described herein, the surprisingly high affinity for lysine makes a thorough characterization of the cooperative allosteric inhibition of this enzyme an instructive model for discriminating between kinetic models for this and other complex enzymatic processes.

MATERIALS AND METHODS

ASA Synthesis. ASA was synthesized according to the reported procedure.[31] Because of the hygroscopic properties of ASA, the concentration of each newly prepared work solution of ASA was determined using the DHDPS−DHDPR coupled kinetic assay described below, in the presence of excess NADH.

Cloning of the dapA and dapB Genes. *C. jejuni* genomic DNA was prepared by Dr. Bonnie Chaban, Western College of Veterinary Medicine, University of Saskatchewan. The *dapA* and *dapB* genes (encoding DHDPS and dihydrodipicolinate reductase (DHDPR), respectively) were PCR-amplified from genomic DNA using KAPA HiFi DNA polymerase (Kapa Biosystems) and forward and reverse primer pairs

5′-GAAAGGGGATCCATGGATAAAAATATTATCATTGGGGC-3′,

5′-ATTCTGCTGCAGTTAAAATCCTTTGATCTTATATTTTTTCATCACTTC-3′ respectively for *dapA*; 5′-TCAAGGGGATCCATGATTAAAATAGGAATTTATGGCG-3′,

5′-ACTGCACTGCAGTTAAATTCCTAAAAAATCATTGATTGAATAC-3′ respectively for *dapB*.

Each pair of genes was ligated into a pQE-80L vector (Qiagen) as *Bam*HI/*Pst*I restriction fragments using T4 DNA-ligase (New England BioLabs). Then *E. coli* XL1-Blue competent cells were transformed with the plasmids. Colonies containing correct recombinant plasmids were identified by analysis of restriction enzymes products, and the DNA comprising the open reading frame of positive candidates was sequenced by the DNA Technologies Unit of the National Research Council, Saskatoon, Canada.

Expression and Purification of Enzymes. TB media[32] (250 mL) containing ampicillin (50 μg/mL) was inoculated with a single colony of *E. coli* XL1-Blue cells containing recombinant plasmid pQE-80L with *dapA* or *dapB* genes and incubated with shaking at 37 °C until cultures reached an OD_{600} of 0.5−0.6. Protein expression was induced by addition of isopropyl β-D-1-thiogalactopyranoside to a final concentration 0.5 mM. The cell culture was incubated with shaking (250 rpm) at 15 °C for 15 h, and then centrifuged at 5180*g* for 30 min. The resulting cell pellet was resuspended in 10 mL of binding buffer (20 mM Tris-HCl, 5 mM imidazole, 500 mM NaCl, 12.5% glycerol, pH 7.9) and sonicated using a Virsonic 600 Ultrasonic Cell Disrupter (VirTis). The sample was chilled in an ice bath during all manipulations. The crude lysate was separated from cell debris by centrifugation at 27000*g* for 30 min at 4 °C. The supernatant containing DHDPS or DHDPR was filtered through a 0.45 μm syringe filter and loaded onto a 1 mL HiTrap IMAC FF column (GE Healthcare), prepared according to the manufacturer's procedure and equilibrated with 5 mL of binding buffer. The column was washed with 5 mL of binding buffer, a 1:1 mixture of binding and washing (20 mM Tris-HCl, 60 mM imidazole, 500 mM NaCl, 12.5% glycerol, pH 7.9) buffers, 10 mL of washing buffer, a 1:1 mixture of binding and stripping (20 mM Tris-HCl, 500 mM NaCl, 100 mM EDTA, 12.5% glycerol, pH 7.9) buffers, and then 15 mL of stripping buffer. Fractions were analyzed by SDS-PAGE, and those containing purified enzyme were pooled and dialyzed at 4 °C for 24 h in storage buffer (20 mM Tris-

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Table 1. Kinetic Constants for *C. jejuni* DHDPS

$K_{M(pyr)}$	$K_{M(ASA)}$	k_{cat}	$k_{cat}/K_{M(pyr)}$	$k_{cat}/K_{M(ASA)}$
0.35 ± 0.02 mM	0.16 ± 0.01 mM	76 ± 1 s^{-1}	(2.2 ± 0.1) × 10^5 M^{-1} s^{-1}	(4.8 ± 0.3) × 10^5 M^{-1} s^{-1}

HCl, 100 mM NaCl, 1 mM DTT, 40% glycerol, pH 7.9). The resulting proteins were concentrated to 0.37 mg/mL for DHDPS and 1.43 mg/mL for DHDPR using an Amicon Ultra-15 Centrifugal filter (30 kDa MWCO, EMD Millipore). Enzyme concentrations were determined by NanoDrop ND-1000 using calculated parameters (ProtParam)[33] (molecular weight and extinction coefficient) for DHDPS (MW 34 069 Da, ε_{280} = 18 068 M^{-1} cm^{-1}) and for DHDPR (MW 28 094 Da, ε_{280} = 15 930 M^{-1} cm^{-1}). The proteins were aliquoted and stored at −80 °C.

Enzyme Assays. The activity of DHDPS was measured using a coupled assay.[3] The initial velocity of the DHDPS enzymatic reaction was determined by monitoring the decrease in absorbance at 340 nm due to oxidation of NADH (ε_{340} = 6220 M^{-1} cm^{-1}). All kinetic measurements were performed on a Beckman DU640 spectrophotometer at 25 °C maintained by circulating water bath. Only freshly prepared solutions of substrates were used in the assay. All measurements were made using 100 mM HEPES buffer at pH 8.0. A typical assay contained 0.16 mM NADH, 0.37 μg of DHDPS (1 μL of stock solution), 7.15 μg DHDPR (5 μL of stock solution), and varying concentrations of ASA (0.60−2.24 mM) and pyruvate (0.12−3.50 mM). The excess amount of DHDPR was determined experimentally, to ensure that the DHDPS-catalyzed reaction would be rate-limiting. A 0.01 M solution of (*S*)-lysine was used in inhibition experiments. Cuvettes containing the assay mixture were incubated for 3 min to equilibrate at 25 °C before the reaction was triggered by the addition of DHDPS. The obtained kinetic data were fitted to the rate eqs 1−4 using Sigma Plot version 10.0:

$$v = V_{max}AB/(K_{M(B)}A + K_{M(A)}B + AB) \quad (1)$$

$$\frac{v}{V_{max}} = \frac{\frac{[S]^n}{K_s^n} + \frac{\beta[S]^n[I]^h}{(\alpha K_i)^h K_s^n}}{1 + \frac{[S]^n}{K_s^n} + \frac{[I]^{h'}}{K_i^{h'}} + \frac{[S]^n[I]^h}{(\alpha K_i)^h K_s^n}} \quad (2)$$

$$\frac{v}{V_{max}} = \frac{\frac{[S]}{K_s} + \frac{\beta[S][I]^h}{K_s K_i^h}}{1 + \frac{[S]}{K_s} + \frac{[S][I]^h}{K_s K_i^h}} \quad (3)$$

$$\frac{v}{V_{max}} = \frac{\frac{[S]^n}{K_s^n} + \frac{\beta[S]^n[I]^h}{K_s^n K_i^h}}{1 + \frac{[S]^n}{K_s^n} + \frac{[I]^h}{K_i^h} + \frac{[S]^n[I]^h}{K_s^n K_i^h}} \quad (4)$$

where V_{max} is the maximum velocity, $K_{M(A)}$ and $K_{M(B)}$ are the Michaelis−Menten constants for two substrates, *A* and *B* are the concentrations of the substrates, *v* is the initial velocity. K_i is the inhibition constant, *I* is the inhibitor concentration, *S* is the substrate concentration, K_S is the dissociation constant of the ES complex, *n* is the Hill coefficient for ASA (when applicable), α and β are proportionality constants, and *h* and *h*′ are Hill coefficients for lysine where, depending on the substrate being bound, *h* and *h*′ are cooperativity coefficients for lysine binding to E and E:pyr or F and F:ASA forms of enzyme respectively in eq 2. Equation 1 is a rate equation for the *ping-pong bibi mechanism*, eqs 2−4 describe *mixed partial*, *uncompetitive partial*, and *noncompetitive partial* mechanisms of inhibition, respectively, to which the Hill coefficients, *h*, *h*′, and *n*, have been introduced. The experiments were conducted by varying concentration of one substrate, while the concentration of the other was saturating or at near-K_M concentration, as indicated in the text.

Equation 5 is the Hill equation for partial inhibition,

$$\log \frac{v_i - v_{i(sat)}}{v_0 - v_i} = \log K - h \log I \quad (5)$$

where v_i is the velocity in the presence of the inhibitor, v_0 is the velocity in the absence of the inhibitor, $v_{i(sat)}$ is the reaction velocity at saturating concentrations of inhibitor, and *K* is an apparent overall dissociation constant. For reactions in which the second substrate was at near-K_m concentration, $v_{i(sat)}$ was measured at [I] = 1.0 mM; for reactions in which the second substrate was at saturating concentration, $v_{i(sat)}$ was measured at [I] = 2.0 mM.

RESULTS

Ping−Pong Mechanism. The kinetic mechanism of catalysis by recombinant histidine-tagged DHDPS from *C. jejuni* was assessed using the DHDPR-coupled assay described above. Note that DHDPR is not selective, in that it can utilize both NADPH and NADH; the latter was used for kinetic studies of DHDPS. Variation of the concentrations of each substrate in the presence of DHDPS and DHDPR resulted in double-reciprocal plots displaying parallel lines, consistent with the canonical ping-pong kinetic mechanism observed for DHDPS from other sources (Supporting Information, Figures S1 and S2). The kinetic constants are shown in Table 1.

Substrate inhibition by ASA, which has been reported for DHDPS from some sources and/or with some ASA preparations,[34] was not observed. The kinetic constants of *C. jejuni* DHDPS at the chosen assay conditions (25 °C, pH 8.0, 100 mM HEPES) are close to those of *E. coli* DHDPS in the same buffer.[17,35] No evidence for significant cooperativity of substrate binding was observed.

Lysine Inhibition. *C. jejuni* DHDPS is strongly inhibited by lysine. A simple observation of the dependence of rate on lysine concentration at fixed substrate concentrations indicates an apparent IC_{50} value near 65 μM (Figure 2). DHDPS from Gram-negative bacteria can be weakly, moderately, or strongly inhibited by lysine showing IC_{50} values from the micromolar to millimolar range: 53 μM for *N. meningitidis*,[16] 0.2 mM for *E. coli*,[36] and 0.7 mM for *S. meliloti*.[37] Figure 2 also reveals two other important features of inhibition. First, the shape of the curve indicates significant cooperativity of inhibition. Second, there is approximately 10% residual activity at saturating concentrations of lysine under the conditions employed. This partial inhibition (or "hyperbolic" inhibition) is also typical of DHDPS from other sources.[13,16,17]

Calculation of Cooperativity Coefficient for Lysine. A series of Hill plots were built over the range from 0.02 to 0.2 mM of lysine, where Hill plots have minimal deviation from linearity, and cooperativity (slopes of Hill plots) can be considered constant. Experimentally obtained Hill plots are normally not linear, and their curvature indicates a mixture of

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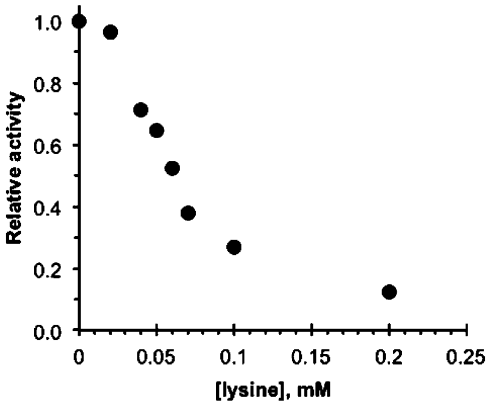

Figure 2. Lysine inhibition of DHDPS activity at 0.16 mM ASA and 3.5 mM pyruvate.

positive and negative cooperativity.[38] By analyzing the shape of Hill plots, it is possible to estimate values of the four intrinsic association constants for the four binding steps of the ligand to the tetramer.[38] When the concentration of inhibitor is low, the complexes with a low value of the Hill coefficient (h) do not contribute significantly to the initial velocity; on the other hand, at high concentrations of the inhibitor it becomes difficult to distinguish small changes in the velocity.[39] Because the inhibition is partial, the logarithm of the ratio "inhibitable activity" in the presence and absence of lysine is plotted versus the logarithm of lysine concentration (eq 5). Values of h are the slopes of the Hill plots. As seen in Figure 3, data collected at

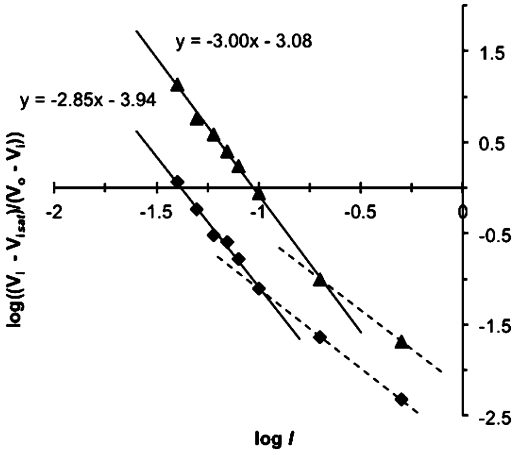

Figure 3. Relationship between degree of cooperativity and concentration of inhibitor. Solid line: linear area (lysine concentration 0.02−0.2 mM). Dashed line: apparent decrease in cooperativity at high lysine concentrations (≥0.2 mM). Data obtained at constant concentration of pyruvate 0.35 mM, 0.072 mM ASA (♦) and 2.0 mM ASA (▲).

concentrations of lysine ≤ 0.1 mM give a straight line, but at higher concentrations the plot deviates from linearity as if value of h were approaching unity, i.e., cooperativity appears to dissipate at very high occupancy of the inhibitory sites. If this were the case, it would manifest itself in biphasic behavior evident from double-reciprocal plots over a wide range of concentrations of lysine.

Results of previous studies of DHDPS inhibition kinetics have differed in some details but consistently have shown that the presence of pyruvate results in tighter binding of lysine to the allosteric site, suggesting that the condensation of pyruvate in the active site somehow alters the nature of the inhibitory site. Because of this, we felt that the degree of cooperativity of lysine binding need not be the same at all substrate concentrations. We therefore conducted experiments to determine cooperativity while varying the concentration of each substrate in the presence of both saturating and near-K_M concentrations of the other (Supporting Information, Figures S3−S6). When the concentration of either substrate was varied in the presence of saturating concentration of the other, the average of h was found to be 2.8 ± 0.2 for both cases. When ASA was maintained at near-K_M concentration (0.18 mM), the value of h averaged 3.0 ± 0.2, and when pyruvate was held at near-K_M concentration (0.35 mM), h = 2.3 ± 0.4. The difference in value at low concentration of pyruvate suggests that this substrate is affecting the cooperativity of lysine binding and is consistent with a kinetic mechanism in which pyruvate binds to the enzyme before lysine does. Drawing conclusions about the kinetic mechanism based on Hill coefficients should be done with caution, however, due in part to experimental error inherent in the calculation of h.

Relationship of Substrate and Inhibitor Binding. Using these Hill plots, we determined the IC_{50} of lysine under a range of conditions. From eq 5, when $v_i = 0.5v_0$, the ordinate equals $\log((0.5v_0 - v_{i(sat)})/(v_0 - 0.5v_0))$ or, after rearranging, $\log(1 - (2v_{i(sat)}/v_0))$. The projection of the ordinate $\log(1 - (2v_{i(sat)}/v_0))$ from the Hill plot on the log I axis gives the IC_{50} value. As shown in Figure 4, IC_{50} values of lysine decrease with

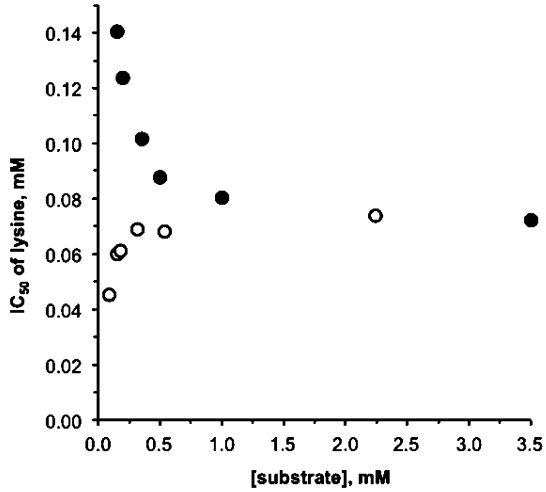

Figure 4. Relationship between IC_{50} of lysine and concentration substrates. (●) Pyruvate is the variable substrate; concentration of ASA is 2.24 mM; (○) ASA is the variable substrate; concentration of pyruvate is 3.50 mM.

increasing pyruvate concentration up to a saturated value. Conversely, IC_{50} values increase with increasing concentration of ASA. This clearly shows that pyruvate *promotes* lysine binding while ASA *hinders* the binding of lysine.

Analysis of Dixon and Cornish−Bowden plots. The Dixon plot ($1/v$ versus I) and the Cornish−Bowden plot (S/v versus I) are very useful to distinguish between kinetic models of simple linear inhibition,[39,40] and when taken together they can provide distinctive patterns that can be attributed to a particular type of inhibition. When these methods are applied to partial inhibition, a difficulty arises from the fact that the Cornish−Bowden plot and the Dixon plot are nonlinear, but

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relatively straight plots can be obtained at concentrations of inhibitor preceding the saturation region. The effect of cooperativity must be taken into account by raising the inhibitor concentration to the power of the Hill coefficient.[40]

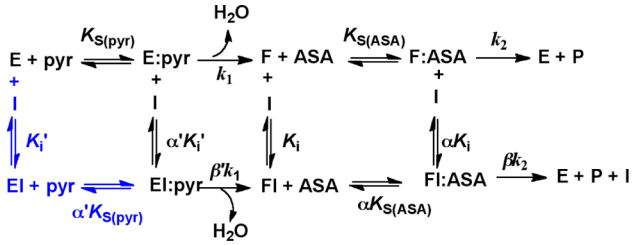
When the concentration of pyruvate was varied at constant [ASA], the plot S/v versus I^h produced a series of lines converging in the second quadrant of the Cartesian coordinate system, and the plot $1/v$ versus I^h yielded a series of near parallel lines (at high concentrations of lysine these lines eventually converge in the first quadrant of the coordinate system), indicative of *uncompetitive* inhibition (Supporting Information, Figure S7A,B). With respect to changing the concentration of ASA, the Cornish–Bowden plot produced a series of lines that cross just below the *x*-axis in the third quadrant, while the Dixon plot produced lines crossing in the second quadrant (Supporting Information, Figure S8A,B). This indicates *mixed* inhibition, with binding of lysine to the F form of the enzyme prevailing over binding to the F:ASA complex. Note for these graphs, the lines were fit independently, rather than trying to fit the data to a single model, to provide an unbiased view of the data. Given the apparent complexity of the inhibition, a global fit of the data is needed for an independent comparison to the diagnostic plots.

Fitting the Inhibition Models to Observed Data. A general scheme illustrating the lysine inhibition mechanism is shown in Scheme 1. For this model, we assumed that the formation of enzyme–pyruvate and modified enzyme–ASA complexes are reversible and rapid steps, and the breakdown of these complexes are slow, rate-limiting steps. Karsten[21] suggested that formation of the final product, i.e., reaction of the F form of the enzyme with the second substrate, ASA, is the rate-limiting step in the overall equilibria; therefore for this scheme $k_2 < k_1$, and our data are consistent with this. The lower part of the scheme illustrates partial inhibition, in which the enzyme–substrate–inhibitor complexes are able to convert the substrates into products at much lower rates compared to reaction rates in the absence of inhibitor ($\beta' < 1$, $\beta < 1$). Assuming that lysine can bind to each form of the enzyme, four K_i values can be determined, and based on the binding affinity of the inhibitor to a particular enzyme species, a decision about the mechanism of inhibition can be made. We have therefore used an unbiased approach and considered all applicable kinetic mechanisms of lysine inhibition with respect to each substrate.

Since binding of lysine is a cooperative process, each rate equation was modified by raising the inhibitor concentration and the inhibition constants to the power of the Hill coefficient (eqs 2–4). Fitting of data was done using cooperativity coefficients as independent variables in the rate equations, and the resulting coefficients were compared with those found by the graphical method. Experimental data obtained by varying the concentration of pyruvate were fit using the mixed partial (hyperbolic) (eq 2) and uncompetitive partial (eq 3) models (Figure 5, Supporting Information, Figures S9–S12). For the *mixed partial* model, the rate constant k_1 (Scheme 1) decreases (by the factor of β'), and the binding affinity of lysine to the E:pyr complex increases (by α'), where $0 < \alpha' < 1$, $0 < \beta' < 1$. Two Hill coefficients, h and h', were introduced into the rate equation to allow for different degrees of cooperativity of lysine binding to the E and E:pyr forms of the enzyme respectively. The cooperativity of pyruvate was equal to 1 (Figures S10 and S12). The *uncompetitive partial* model describes the situation when inhibitor produces its effect by binding only to the enzyme–substrate complex, the series of double-reciprocal plots are not parallel and the limiting plot at $I = \infty$ is a horizontal line (Figure 5, Supporting Information, Figures S9 and S11). Both regression models describe the experimental data well (R^2 close to 1) (Supporting Information, Table S1). For the mixed partial model, several parameters were poorly estimated: the cooperativity of lysine binding to the E:pyr complex (h exceeds the number of binding sites in the tetramer), and K_i' and α' (have high statistical error) (Supporting Information, Table S1). On the basis of the information obtained, including R^2 values, distribution of residuals, and shape of secondary plots (slope and intercept vs lysine concentration plots are not shown), preference should be given to the *uncompetitive partial* model as the model providing a good fit with fewer variables.

The most common conclusion in the recent literature is that (*S*)-lysine is a partial noncompetitive inhibitor of DHDPS with respect to ASA,[14,16,17] which implies that lysine is able to bind to both F enzyme form (pyruvate-bound) and F:ASA complex with the same affinity: $K_i = \alpha K_i$ (Scheme 1). Assuming that the binding affinities could be different, the model of mixed partial inhibition ($1 < \alpha < \infty$, $0 < \beta < 1$) was considered as well. The family of double-reciprocal plots ($1/v$ versus $1/[ASA]$ at

Scheme 1. General Scheme of DHDPS Lysine Inhibition[a]



[a]The equilibria shown in blue are not observed for *C. jejuni* DHDPS. For this general scheme, there is no limit on the values of α (and α'), while β (and β') must be <1 for inhibition. In a purely noncompetitive partial inhibition mechanism, α and $\alpha' = 1$, whereas in a mixed partial inhibition mechanism, α and $\alpha' > 1$.

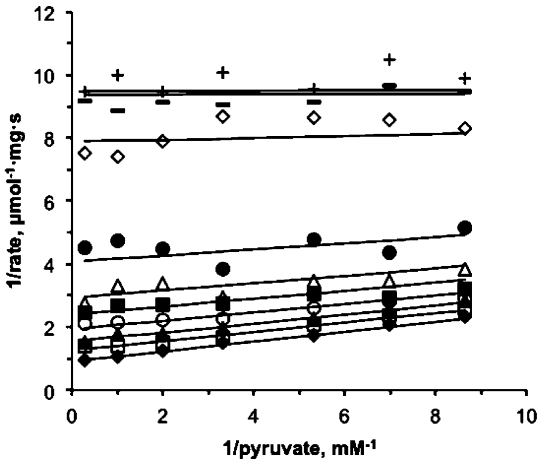


Figure 5. Double-reciprocal plot of data obtained at a constant ASA concentration of 0.18 mM. Concentration of lysine: (♦) 0 mM, (□) 0.04 mM, (▲) 0.05 mM, (○) 0.06 mM, (■) 0.07 mM, (Δ) 0.08 mM, (●) 0.10 mM. (−) 0.20 mM, (◇) 0.50 mM, (+) 1.00 mM. Solid lines are fit lines, obtained by global fitting the *uncompetitive partial* model to the data. Residuals are shown in Supporting Information Figure S9.

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different lysine concentrations) has a mutual point of convergence near the *y*-axis when [lysine] ≤ 0.1 mM. At high lysine concentration, plots have a limiting slope and look more like a set of parallel lines (Figure 6). Taking into account that

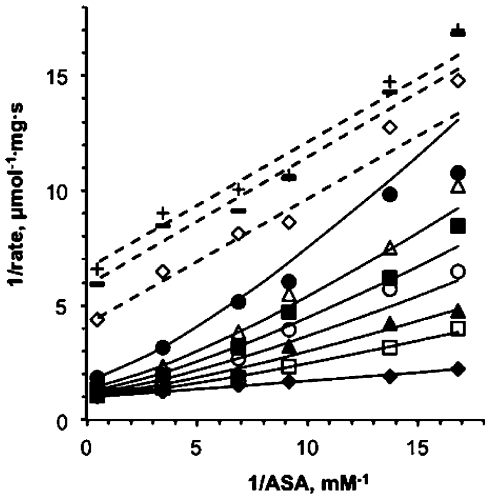

Figure 6. Double-reciprocal plot of data obtained at a constant pyruvate concentration of 0.35 mM fit to the mixed partial inhibiton model (eq 2, $1 < \alpha < \infty$, $0 < \beta < 1$). Solid lines, Hill coefficient for ASA $n = 1.3$; dashed lines, $n = 1$. Concentration of lysine: (♦) 0 mM, (□) 0.04 mM, (▲) 0.05 mM, (○) 0.06 mM, (■) 0.07 mM, (Δ) 0.08 mM, (●) 0.10 mM, (−) 0.20 mM, (◇) 0.50 mM, (+) 1.00 mM.

the apparent K_M values for ASA increase at lysine concentrations less than 0.1 mM, and then slightly decrease at higher lysine concentrations, it can be concluded that the kinetic mechanism changes at saturating concentrations of lysine.

The analysis of the mechanism of lysine inhibition with respect to ASA is more complex. The double-reciprocal plot in Figure 6 again indicates two distinct conditions: at low lysine concentrations (≤0.1 mM), a family of intersecting lines is evident, as is detectable curvature of the lines; at higher lysine concentrations the lines appear parallel, with no apparent curvature. Although there are several arguments that might explain a change in behavior of an enzyme saturated with an allosteric ligand, the curvature of the lines suggests that there is lysine-induced cooperativity of ASA under some conditions. Construction of Hill plots indicated that the cooperativity coefficient for ASA is a nonlinear function of lysine concentration (Figure 7). In the presence of lysine, and at near-K_M concentration of pyruvate, ASA binds with an average cooperativity coefficient $n = 1.3 \pm 0.3$, and this value was therefore included in eqs 2 and 4, generating the lines of fit included in Figure 6 at lysine concentrations ≤0.1 mM. At saturating concentration of pyruvate, the average cooperativity coefficient for ASA is close to 1 (1.1 ± 0.3).

Regression analysis of the data indicated that while both noncompetitive partial (Figures S13 and S14) and mixed partial (Figures 6, S16, S15) models approximate the data, the mixed partial model has better statistical characteristics. The statistical difference is small, but analysis of residual plots revealed that in the case of the noncompetitive partial model (at near-K_M concentration of pyruvate), residuals are distributed non-randomly (Figure S13B). When combined with the examination of the Cornish−Bowden and Dixon plots, this indicates that lysine is a *mixed partial* inhibitor with respect to ASA,

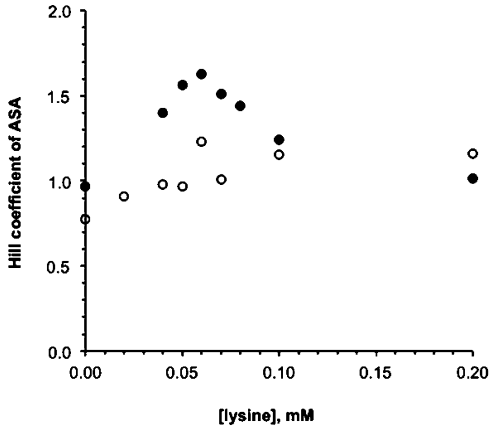

Figure 7. Values of the Hill coefficient of ASA at varying concentrations of (*S*)-lysine and constant pyruvate concentrations, 0.35 mM (●) and 3.50 mM (○).

where the inhibitor binds with higher affinity to the F form of the enzyme and with lower affinity to the F:ASA complex.

The lysine inhibition results are summarized in Table 2. The values of dissociation constants, $K_{S(pyr)}$ and $K_{S(ASA)}$, are close to the K_M values for these substrates in Table 1 (for near-K_M background concentration of the second substrate, K_S values are approximately half of those for saturating concentration of the second substrate), which suggests that the assumptions that have been made about rapid and slow steps in the overall equilibria (Scheme 1) are correct.

DISCUSSION

Inhibition of DHDPS from various organisms has been studied, and the results of these studies have demonstrated three challenges: defining the kinetic mechanism of inhibition; understanding exactly how the binding of lysine to the regulatory site results in impaired catalysis; and the design of potent, selective inhibitors of the enzyme. The first of these challenges is addressed here for *C. jejuni* DHDPS.

Studies on the *E. coli* enzyme show that the active site contains a lysine residue, Lys161, with which pyruvate condenses in the first chemical step of the ping−pong mechanism. The reaction apparently relies on a "catalytic triad"[35] of Thr44, Tyr133, and Tyr107′ (a residue contributed from the other polypeptide chain of the tight dimer). Arg138 has been shown to be important for ASA binding to the enzyme.[41] These residues are present in *C. jejuni* DHDPS as Lys166, Thr47, Tyr137, Tyr111′, and Arg142. Moreover, the structure of *E. coli* DHDPS with lysine bound in the active site shows each lysine molecule making polar contacts with seven residues of the protein (Ser48, Ala49, Leu51, His56, Asn80′, Glu84′, and Tyr106), and, as shown in Figure 8, all of these residues are conserved in the allosteric site of *C. jejuni* DHDPS (Ser51, Ala52, Leu54, His59, Asn84′, Glu88′, and Tyr110). In short, it would be reasonable to expect these homologues to display very similar behavior in catalysis and inhibition. We were therefore surprised to find distinct differences in the kinetic mechanism of inhibition, as demonstrated in independent kinetic analyses of the data: we observe that lysine is an uncompetitive partial inhibitor with respect to pyruvate, and a mixed partial inhibitor with respect to ASA. Kinetic mechanisms can vary among enzymes from different species, although some differences in our results may be attributed to our treatment of cooperativity in the mechanistic analysis,

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Table 2. Lysine Inhibition Kinetic Parameters for *C. jejuni* DHDPS

kinetic model	uncompetitive partial		mixed partial	
variable substrate	pyruvate		ASA	
fixed substrate	ASA (sat.)	ASA (K_M)	pyruvate (sat.)	pyruvate (K_M)
$h_{E:pyr}$, lysine	2.6 ± 0.1	2.9 ± 0.1		
h_F, lysine			2.3 ± 0.2	2.2 ± 0.1
$h_{F:ASA}$, lysine			2.8 ± 0.2	3.2 ± 0.2
n, ASA			1.1 ± 0.3	1.3 ± 0.3
K_{i1}, mM[a]	0.069 ± 0.001	0.054 ± 0.001		
K_{i2}, mM[b]			0.045 ± 0.003	0.037 ± 0.001
K_{i3}, mM[c]			0.072 ± 0.009	0.100 ± 0.006
$K_{S\ (ASA)}$, mM			0.12 ± 0.01	0.073 ± 0.003
$K_{S\ (pyr)}$, mM	0.33 ± 0.01	0.17 ± 0.01		

[a] K_{i1} corresponds to $\alpha' K_i'$ in the Scheme 1. [b] K_{i2} corresponds to K_i in the Scheme 1. [c] K_{i3} corresponds to αK_i in the Scheme 1.

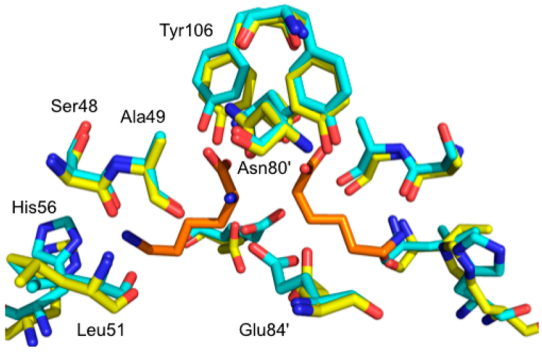

Figure 8. Overlay of the allosteric site residues of *C. jejuni* DHDPS (cyan), and *E. coli* DHDPS (yellow) making polar contacts with bound L-lysine (orange). Residue labels indicate *E. coli* residues making contact with one L-lysine molecule. Coordinates from RSCB PDB entries 1YXD (*E. coli*) and 3LER (*C. jejuni*). Figure generated using PyMOL Molecular Graphics System, Version 1.4.1, Schrodinger, LLC.

which was absent from recent treatments of the *E. coli* DHDPS mechanism. In arriving at our conclusions, we found the use of the combination of Cornish−Bowden and Dixon plots to be extremely helpful in differentiating the kinetic models.

Binding of lysine to DHDPS has been shown to be cooperative for DHDPS from other sources, including *E. coli*,[21,24] *S. meliloti*,[25] and *V. vinifera*.[23] It is therefore surprising that although this cooperativity has been known for 15 years, most detailed kinetic analyses have not accounted for cooperativity. (During the preparation of this manuscript, a report describing the kinetics of inhibition of the *V. vinifera* DHDPS appeared, in which Hill coefficients were calculated and incorporated into rate equations.) In some cases perhaps, lysine cooperativity is small enough to be ignored, but the significant cooperativity evident with *C. jejuni* DHDPS made it clear that Hill coefficients would have to be incorporated into our analyses.

As described above, the degree of cooperativity was not the same in all conditions. This is not surprising: the presence of pyruvate increases the affinity of the allosteric site for lysine, while the presence of ASA decreases the affinity of the allosteric site for lysine. In each case, the properties of the site have changed, albeit subtly, and therefore there is no reason for the degree of cooperativity to remain constant. In all conditions, the value of h was between 2 and 3.2. That the degree of cooperativity exceeds 2 is significant in that it indicates that the two allosteric sites are not entirely independent; binding of lysine to one site affects the binding of lysine at the other, showing the enzyme to be functioning as a tetrameric catalyst, rather than as a dimer of independent dimers. It is unlikely that this site-to-site communication is due to a pronounced structural change, since no evidence has been observed for such changes in the very similar *E. coli* DHDPS. Dobson et al.[30] and Reboul et al.[42] have used molecular dynamics simulations to show the likely importance of protein dynamics to DHDPS catalysis, and the role that the tetrameric structure may play in maintaining optimal active site residue conformation.

The complexity of the inhibition mechanism extends beyond the cooperativity of inhibitor binding. The behavior of the enzyme at high concentrations of lysine can be differentiated from its behavior at lysine concentrations below 0.1 mM. Specifically, the Lineweaver−Burk plots of Figure 6 show a different pattern, with the lines at high lysine concentrations near parallel. This can be understood in terms of Scheme 1 by considering that at high lysine concentrations, catalysis passes largely through the lower pathway, i.e., the inhibitor-bound enzyme species. We also observe that the presence of lysine induces cooperativity in the second substrate, ASA. It may be that the presence of lysine restricts or alters the dynamics of the protein, which thereby alters several properties of catalysis, such as cooperativity of substrate binding.

In summary, although the kinetic mechanism of inhibition is complex, there are some clear conclusions to be drawn from these results. Lysine is a more effective inhibitor of *C. jejuni* DHDPS than of most DHDPS enzymes from Gram-negative bacteria, and the observed kinetic mechanism differs from that reported recently for DHDPS from other sources. It is evident that the inhibition mechanism cannot be assessed properly without accounting for the cooperativity of lysine binding. The value of the Hill coefficient with respect to each binding event varies somewhat, but in all cases $h > 2$, indicating that the two allosteric sites are not independent. Lysine binds most effectively to the F form of *C. jejuni* DHDPS, and kinetic results suggest that lysine either does not bind to the E form (this equilibrium is shown in blue in Scheme 1) or binding of lysine to this form does not have an effect on inhibition kinetics. Figure 4 clearly shows that the presence of pyruvate promotes lysine binding, while ASA and lysine have an antagonistic relationship; the presence of ASA drives up the apparent inhibition constant of lysine, and the presence of lysine drives up the apparent Michaelis constant of ASA; each hinders the binding of the other. Finally, this is the first observation of lysine-induced cooperativity of ASA. As

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observed previously for other DHDPS enzymes, there is no cooperativity in the absence of lysine, but the curvature of the lines in Figure 6 clearly demonstrates the low but significant cooperativity of the second substrate. The specifics of the signal transduction between active site and allosteric site, and between antipodal allosteric sites, remain unclear, but will be aided by biophysical studies of this protein including high-resolution crystallographic studies.

■ ASSOCIATED CONTENT

Supporting Information

Additional kinetic plots, table comparing results with alternative kinetic models, SDS-PAGE of purified proteins. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

ASA, (*S*)-aspartate β-semialdehyde; DHDPS, dihydrodipicolinate synthase; DHDPR, dihydrodipicolinate reductase

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