Rapid Reports

Isothermal Titration Microcalorimetry Reveals the Cooperative and Noncompetitive Nature of Inhibition of *Sinorhizobium meliloti* L5-30 Dihydrodipicolinate Synthase by (*S*)-Lysine[†]

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ABSTRACT: MosA, a dihydrodipicolinate synthase (DHDPS) from *Sinorhizobium meliloti* L5-30, catalyzes a class I aldolase reaction that is allosterically inhibited by (*S*)-lysine. The thermodynamics of (*S*)-lysine binding to apoenzyme, and to enzyme saturated with pyruvate or with 2-oxobutyrate, are evaluated here using isothermal titration microcalorimetry. Results unambiguously support a noncompetitive mechanism, with substrate-dependent differences in the energetics of inhibitor binding. Inhibition is strikingly cooperative: a second molecule of (*S*)-lysine binds 10^5 times more tightly than the first.

Dihydrodipicolinate synthase (DHDPS,[1] EC 4.2.1.52) is a class I aldolase that catalyzes the condensation of pyruvate with aspartate-β-semialdehyde (ASA) to produce (*S*)-4-hydroxytetrahydrodipicolinic acid (HTPA), which spontaneously dehydrates to dihydrodipicolinate, an intermediate found in the (*S*)-lysine biosynthetic pathway in bacteria and plants. Pyruvate condenses with an active site lysine residue (Lys161 in MosA) (*1*) to form a Schiff base, which acts as an electron sink for enamine formation. Binding of ASA then allows the aldol reaction, followed by transimination to form HTPA (*2*). DHDPS enzymes have garnered interest as targets for antimicrobial and herbicidal compounds (*3, 4*) and as a means of producing lysine-rich crops (*5*).

(*S*)-Lysine is a feedback inhibitor of DHDPS from plants and certain bacteria. Typically, DHDPSs from Gram-negative bacteria are weakly inhibited, with IC_{50} values of 0.1–0.5 mM (*6, 7*). Extensive kinetic and structural studies of *E. coli* DHDPS determined that it follows a substituted-enzyme (also called ping-pong or modified-enzyme) kinetic mechanism and that (*S*)-lysine is an allosteric, cooperative inhibitor of the enzyme (*8, 9*). Crystal structures of the homotetrameric DHDPS from *E. coli* have revealed that the (*S*)-lysine binding site is located at a monomer−monomer interface, where two molecules of (*S*)-lysine bind such that each one makes contact with both monomers. A water-filled channel is found between the active site and the (*S*)-lysine binding site in which the first (*S*)-lysine makes up part of the binding site of the second (*S*)-lysine (*10*). The nature of the inhibition was originally reported to be uncompetitive with respect to pyruvate (*9*), based on the family of parallel lines evident from double-reciprocal plots at varying concentrations of (*S*)-lysine. The explicit conclusion from this result is that (*S*)-lysine binds only to the F-state of the enzyme, i.e., after binding and reaction of pyruvate. However, Gerrard (*2*) pointed out that because DHDPS is not 100% inhibited by (*S*)-lysine, the inhibition is hyperbolic, and therefore (*S*)-lysine is a mixed noncompetitive inhibitor that can bind to the free enzyme. This is an important distinction: there are no crystal structures of DHDPS liganded by both (*S*)-lysine and substrate. If (*S*)-lysine did not bind to the free (E-state) enzyme, then the DHDPS−lysine complex could be viewed as an artifact. Despite considerable kinetic and structural information available on DHDPS enzymes, it is not clear how binding of (*S*)-lysine inhibits the reaction.

MosA is a controversial DHDPS orthologue from *Sinorhizobium meliloti* L5-30. First suggested to be an *O*-methyltransferase required for rhizopine biosynthesis (*11–13*), MosA has been shown by our laboratory to be a DHDPS, feedback-inhibited by (*S*)-lysine (*14*), possessing no *O*-methyltransferase activity (*1*). We used isothermal titration microcalorimetry (ITC) to detect and evaluate interactions between MosA and pyruvate, revealing this process to be largely entropically driven. MosA shares a high degree of sequence identity with *E. coli* DHDPS (~45%), and the high-resolution crystal structure, consistent with our kinetics experiments, shows no significant differences between these two enzymes. The lack of a clear picture of the nature of inhibition by (*S*)-lysine led us to use ITC to observe the binding of this inhibitor directly.

We performed ITC experiments titrating (*S*)-lysine into buffered solutions of MosA in the presence or absence of saturating amounts of pyruvate. Using Bindworks, a cooperative binding model fit the data well, consistent with prior

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[1] Abbreviations: ASA, L-aspartate-β-semialdehyde; DHDPS, dihydrodipicolinate synthase; HTPA, (*S*)-4-hydroxytetrahydrodipicolinic acid; ITC, isothermal titration microcalorimetry.

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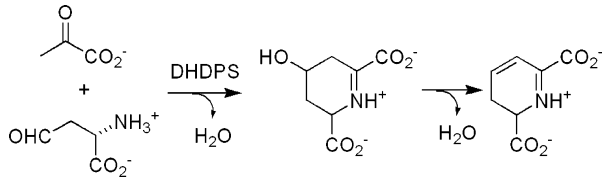

FIGURE 1: Reaction catalyzed by DHDPS MosA.

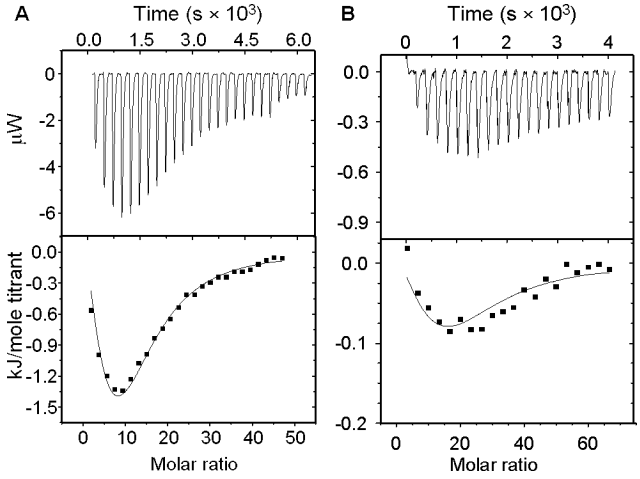

FIGURE 2: (A) ITC titrations of (*S*)-lysine into pyruvate/MosA. The top graph shows raw data for 20 injections (5 μL) of (*S*)-lysine (50 mM) into a buffered solution of MosA (0.06 mM based on dimer molar mass) saturated with pyruvate at 25 °C. The bottom graph shows data points as energy as a function of molar ratio; the solid line represents the fit to the cooperative binding model from Bindworks 1.0. (B) Same experimental procedure, using MosA (0.5 mM dimer) in the absence of pyruvate.

kinetic data supporting the cooperativity of (*S*)-lysine binding to Gram-negative DHDPSs. Furthermore, models describing independent or multiple independent models did not result in reasonable fits to the data.

A typical isotherm produced by titrations of (*S*)-lysine into MosA saturated with pyruvate is shown in Figure 2A. Experimental methods have been described previously (*1*). The curve is clearly diagnostic of cooperative binding: addition of ligand in the early stages results in increasing heats of binding, followed by saturation of the protein by ligand. Thermodynamic values for the first and second binding events were determined from these data (Table 1). (*S*)-Lysine initially binds with very weak affinity; slightly favorable enthalpy was observed, and a small change in entropy was calculated. The second molecule of (*S*)-lysine binds much more tightly, displaying significant favorable changes in enthalpy and entropy. Titration of (*S*)-lysine into a solution of pyruvate in imidazole buffer showed small, uniform dilution heats, indicating the results are due to specific interaction with MosA (data not shown).

A second set of experiments were conducted to observe directly whether (*S*)-lysine is a mixed or uncompetitive inhibitor of MosA. Binding of inhibitor to protein in the absence of substrate necessarily indicates binding to the E-state of the enzyme (i.e., the apoenzyme), characteristic of a mixed-inhibition mechanism. Isotherms (Figure 2B) clearly show an interaction between MosA and (*S*)-lysine occurs without pyruvate bound to enzyme. Although the experimental data show an increased variance from the calculated curve, the binding remained cooperative, and the values were reproducible. Thermodynamic data obtained (Table 1) reveal no significant differences in K_{d1}or K_{d2} in the absence of pyruvate. Furthermore, the enthalpy and entropy values extracted for $\Delta H_{1(Pyr)}$ and $\Delta H_{1(Apo)}$ as well as $T\Delta S_{1(Pyr)}$ and $T\Delta S_{1(Apo)}$ displayed no significant differences. However, significant changes in the enthalpy and entropy of binding of the second inhibitor are seen such that a complete reversal of the dominating forces of binding is observed.

The enthalpy change for binding of the second (*S*)-lysine drops sharply, while the entropy change increases in a compensatory fashion (Table 1). Since the enthalpy term for the binding of the second lysine is much smaller, i.e., has become less favorable, there is apparently a loss of some important intermolecular interactions during the binding event in the absence of pyruvate. This, however, was offset by an increase in the entropy; therefore, the affinity constants (K_{d2}) were essentially unchanged in the absence and presence of pyruvate. Clearly, the association between (*S*)-lysine and MosA is heavily influenced by the presence of pyruvate in the catalytic site.

Further experimental evidence for some form of communication between the active site and the (*S*)-lysine binding site is found from titrations using MosA saturated with 2-oxobutyrate (Table 1). We have demonstrated that 2-oxobutyrate forms a Schiff base with MosA in a manner analogous to that for pyruvate (*1*). The thermodynamic parameters extracted for binding of the first lysine remain relatively unchanged, but the K_{d2} value is only slightly greater while the enthalpy and entropy values are between those determined for the second (*S*)-lysine in the presence and absence of pyruvate. Figure S1 (Supporting Information) shows that the fit of the data to a cooperative binding model is not as good as in the case of Figure 2, but alternative models did not fit the data. The binding events in the presence of 2-oxobutyrate may not be fully described by this model. These data support the proposal that interactions are occurring with 2-oxobutyrate or pyruvate in the active site of MosA that are influencing the thermodynamics of (*S*)-lysine binding.

Changes in heat capacity provide useful information about the release of solvent upon binding of a ligand to a protein (*16*). Unlike enthalpy, entropy, and free energy, heat capacity changes can distinguish between effects caused by polar and nonpolar hydration (*17*). Titrations of (*S*)-lysine into pyruvate-saturated solutions of MosA at 15, 18, 20, and 25 °C revealed a large positive heat capacity (ΔC_p) of approximately 1 kJ mol^{-1} K^{-1} (see Figure S3 of the Supporting Information), indicating the importance that solvent plays in the binding of (*S*)-lysine to MosA (see Table S1 of the Supporting Information). Large positive heat capacity changes are rare for associations involving proteins but are well-documented (*18–21*). During the association of (*S*)-lysine with MosA, the burial of a charged, polar molecule into a polar, charged binding site on MosA would contribute positively to ΔC_p. The crystal structure of *E. coli* DHDPS bound to (*S*)-lysine (*10*) shows each inhibitor molecule interacting with the polar side chains of Tyr106, Asn80′, and Glu84′, among other residues, all of which are conserved in MosA (*14*). Additionally, the inhibitor binding site on MosA would also require desolvation prior to the binding of the second inhibitor and thereby contribute positively to the ΔC_p values.

Table 1: Thermodynamic Data for (*S*)-Lysine Binding to MosA

enzyme form	K_{d1} (M)	K_{d2} (μM)	ΔH_1 (kJ/mol)	ΔH_2 (kJ/mol)	ΔG_1 (kJ/mol)	ΔG_2 (kJ/mol)	$T\Delta S_1$ (kJ/mol)	$T\Delta S_2$ (kJ/mol)
MosA and pyruvate[a]	0.4 ± 0.1	2.2 ± 0.7	-2.2 ± 0.4	-21 ± 2	-2.3 ± 0.6	-32 ± 10	0.2 ± 0.7	11 ± 10
apo-MosA[b]	0.4 ± 0.1	3.2 ± 0.7	-0.8 ± 0.5	-3.3 ± 0.1	-2.5 ± 0.6	-31 ± 7	2 ± 1	28 ± 7
MosA and 2-oxobutyrate[a]	0.3 ± 0.1	10 ± 3	-0.3 ± 0.1	-14 ± 1	-3.4 ± 1	-28 ± 9	3 ± 1	14 ± 9

[a] Experiments performed in imidazole buffer (100 mM, pH 7.7) at 25 °C with 10 mM pyruvate or 10 mM 2-oxobutyrate. Values are an average of at least three independent trials ± the standard deviation unless otherwise indicated. [b] Values are an average of two independent trials ± the standard deviation.

Protein conformational changes upon association of a ligand often result in increased exposure of residues to the solvent, leading to a positive ΔC_p as seen with some antibody−antigen interactions (*22*). Increasing the surface area of hydrophobic residues accessible to water during a binding event would increase the magnitude of ΔC_p (*15*). Changes in protein structure that lead to an increase in the number of "soft internal vibrations" (internal degrees of freedom with force constants low enough to be affected significantly by changes to their environment) will also contribute positively to heat capacity (*16, 21*). One or more of these factors, desolvation of the charged ligand and protein residues, exposure of nonpolar residues to solvent, and an increase in the number of easily accessible vibration states, may lead to the large observed positive heat capacity.

In the case of DHDPS from *E. coli*, crystallographic data of the enzyme with bound inhibitor did not reveal large conformational changes, although several small changes were noted (*10*). However, the crystal structure determined was that of *E. coli* DHDPS bound to (*S*)-lysine without pyruvate present. It is interesting to note that no crystal structure has been determined for a DHDPS bound to both pyruvate and (*S*)-lysine. (This is not for lack of effort on the part of us or, in all likelihood, others.) ITC experiments clearly illustrate the thermodynamic differences upon binding of (*S*)-lysine to MosA in the presence and absence of pyruvate. Consequently, inferences made regarding the interaction (*S*)-lysine with apo-DHDPS may only provide part of the story of how the enzyme is inhibited. Allosteric inhibition is often rationalized by a conformational change of the enzyme induced upon binding of inhibitor. In the case of DHDPS MosA, since no significant change is apparent between the pyruvate-bound and (*S*)-lysine-bound enzyme, it may be that (*S*)-lysine does not allow necessary conformational dynamics associated with the binding and/or reaction of the second substrate, ASA.

We conclude that (*S*)-lysine is a noncompetitive inhibitor of MosA with respect to pyruvate, that the presence of substrate has a substantial effect on the nature of enzyme–inhibitor association, and that solvent plays a key role in binding of inhibitor. A molecule of (*S*)-lysine binds very loosely, creating a partially filled site to which the second (*S*)-lysine binds so tightly that binding would be nearly simultaneous. This suggests an effective strategy for inhibition of the enzyme could be based on compounds designed to mimic a pair of bound lysine molecules.

Finally, there may be many cases in which inhibitor solubility or other factors prevent determination of hyperbolic inhibition by kinetic methods. ITC has been shown to be an effective method for determining the type of inhibition, one that paints a vivid picture of the binding events.

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SUPPORTING INFORMATION AVAILABLE

Experimental procedure for ITC, ITC of (*S*)-lysine into a MosA solution containing 10 mM 2-oxobutyrate, temperature dependence of enthalpy of the second (*S*)-lysine binding to MosA with pyruvate, table of the temperature dependence of thermodynamic parameters. This material is available free of charge via the Internet at http://pubs.acs.org.

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