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The Slow-Onset Nature of Allosteric Inhibition in α-Isopropylmalate Synthase from *Mycobacterium tuberculosis* Is Mediated by a Flexible Loop

Ashley K. Casey, Joshua Baugh, and Patrick A. Frantom*

Department of Chemistry, The University of Alabama, Tuscaloosa, Alabama 35487, United States

Supporting Information

ABSTRACT: The identification of structure−function relationships in allosteric enzymes is essential to describing a molecular mechanism for allosteric processes. The enzyme α-isopropylmalate synthase from *Mycobacterium tuberculosis* (MtIPMS) is subject to slow-onset, allosteric inhibition by L-leucine. Here we report that alternate amino acids act as rapid equilibrium noncompetitive inhibitors of MtIPMS failing to display biphasic inhibition kinetics. Amino acid substitutions on a flexible loop covering the regulatory binding pocket generate enzyme variants that have significant affinity for L-leucine but lack biphasic inhibition kinetics. Taken together, these results are consistent with the flexible loop mediating the slow-onset step of allosteric inhibition.

The improvement in experimental ability to detect conformational ensembles in proteins has largely affected our description of catalytic and allosteric mechanisms in enzymes. The ability of an allosteric effector to modulate an enzyme's conformational population (or subpopulations within a single conformational well) has emerged as a viable mechanism for allostery.[1−3] Over the past decade, multiple computational and experimental techniques for mapping regions of a protein that are perturbed upon effector binding have been developed, providing support for this mechanism.[4−6] Regardless of the phenomenological mechanism of allostery, a long-term goal of the field is a molecular description of specific structure−function relationships responsible for communicating the allosteric signal. An understanding of these relationships is necessary to exploit this mechanism for development of allosteric therapeutics or engineered allosteric enzymes for biosensors or industrial microbial strains.

In opposition to a traditional two-state model of allostery, a prediction of a conformational population model is that multiple forms of an inhibited enzyme−effector complex are possible. Allosteric effectors that act through a two-step, slow-onset kinetic mechanism exemplify this prediction by including two conformations of the enzyme−effector complex. Within the framework of conformational ensembles, slow-onset allosteric effectors can be described by an equilibrium between two conformations of the enzyme−effector complex with different kinetic properties separated by an activation barrier that is similar in magnitude to the barrier for the rate-limiting step in the reaction. While this description is thermodynamically identical to a traditional competitive slow-onset inhibitor, it should be noted that an allosteric inhibitor would not be capable of converting transition-state stabilization energy into binding energy, as is proposed for competitive inhibitors.[7] Kinetic models describing hysteretic behavior in enzymes have also been described;[8] however, the fundamental mechanism of slow-onset allosteric inhibitors remains unclear.

The enzyme α-isopropylmalate synthase (IPMS) catalyzes the first step in the biosynthesis of L-leucine in archaea, bacteria, and some eukaryotes. IPMS catalyzes a Claisen condensation between acetyl coenzyme A (AcCoA) and α-ketoisovalerate (KIV) to form isopropylmalate and CoA. The enzyme has been isolated from multiple species and is subject to feedback inhibition by L-leucine.[9] Because of its role as a possible drug target, IPMS from *Mycobacterium tuberculosis* (MtIPMS) has been extensively characterized. Structurally, MtIPMS is a homodimer with each monomer consisting of an N-terminal catalytic domain, a flexible linker domain, and a C-terminal regulatory domain (Figure 1A).[10] MtIPMS requires a divalent metal for activity and is activated by monovalent cations.[11] Importantly, MtIPMS is feedback inhibited by L-leucine through a reversible two-step, slow-onset mechanism,[12]

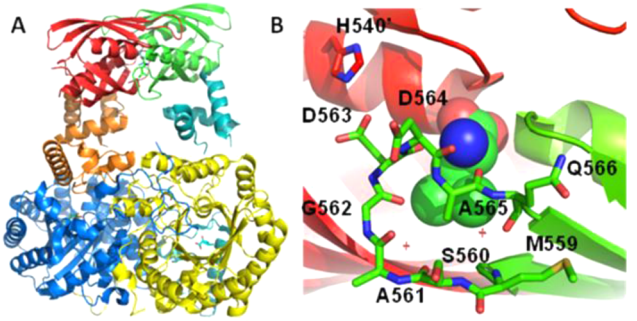

Figure 1. Three-dimensional structure of MtIPMS. (A) Ribbon structure of the dimeric enzyme. Catalytic domains are colored blue and yellow; the linker is colored teal and orange and the regulatory domain green and red. (B) Leucine binding site at the interface of the regulatory domains. Residues on the flexible loop are represented as sticks; leucine is shown in space-filling mode, and two ordered water molecules are shown as red asterisks. Both structures were created from Protein Data Bank entry 3hps and rendered with PyMol.

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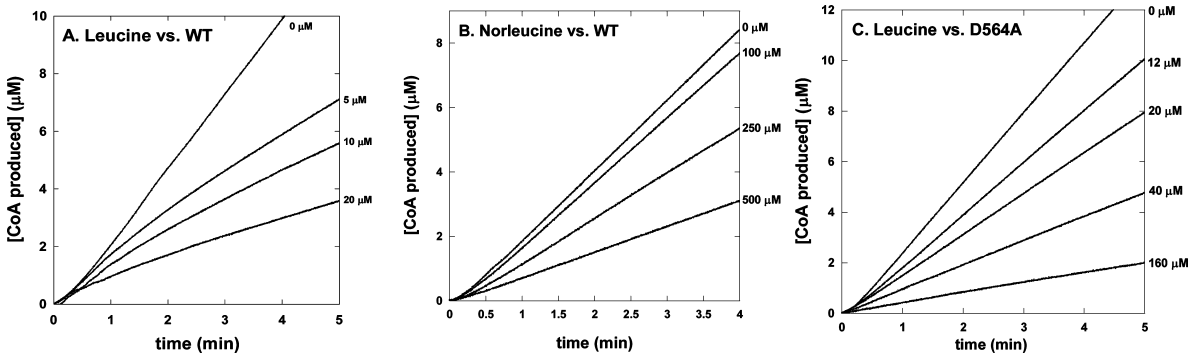

Figure 2. Example progress curves displaying linear kinetics for wild-type MtIPMS vs leucine and norleucine and D564A MtIPMS vs leucine. Solid lines show the measured absorbance at 324 nm in the continuous assay described in the Supporting Information. The concentrations listed are the L-leucine concentrations in each assay.

resulting in biphasic progress curves in the presence of L-leucine (Figure 2A), adding it to a small group of documented allosteric systems displaying such behavior.[13−15] L-Leucine acts as a V-type inhibitor, affecting the k_{cat} of the enzyme without perturbing its affinity for the substrates. Two molecules of L-leucine bind to the dimeric enzyme; however, there is no evidence of cooperativity.[12,16]

Previous structural studies indicate that upon L-leucine binding, a flexible loop covering the binding site becomes more ordered (Figure 1B); however, no structural changes consistent with impaired activity are found in the catalytic domain.[10] The results of solution-phase backbone amide hydrogen−deuterium exchange are also consistent with changes in the backbone dynamics of the regulatory domain upon L-leucine binding.[5] L-Leucine makes polar contacts between backbone atoms in the regulatory domain, while the hydrophobic side chain interacts with multiple residue side chains, including several on the flexible loop. Here, we report two separate lines of evidence that identify the flexible loop as a major determinant in the slow-onset nature of the inhibition.

The amino acids norvaline, norleucine, methionine, and isoleucine were tested as possible allosteric inhibitors of MtIPMS. All of the amino acids are capable of inhibiting MtIPMS activity, though none as efficiently as L-leucine (Table S1 of the Supporting Information). Initial velocity plots were consistent with alternate amino acids acting as noncompetitive inhibitors, similar to L-leucine (Figure S1 of the Supporting Information). L-Norvaline and L-norleucine were the most effective inhibitors with K_i values ranging from 200 to 400 μM. Interestingly, MtIPMS displays linear kinetics in the presence of the alternate amino acid inhibitors (representative data for L-norleucine shown in Figure 2B).

One explanation for the loss of slow-onset kinetics is that the altered side chains of the amino acids perturb the closure of the flexible loop over the regulatory binding site. To test this hypothesis, we used site-directed mutagenesis to create enzyme variants with substitutions at residues on the flexible loop (Figure 1B). All of the non-alanine and -glycine residues in the loop and His540 were subjected to alanine scanning mutagenesis. All of the enzyme variants were soluble and purified using conditions identical to those used for the wild-type enzyme. All enzyme variants were determined to be dimeric on the basis of the results of size exclusion chromatography. Kinetic parameters were determined for each enzyme variant (Table S2 of the Supporting Information). Other than S560A MtIPMS, all enzyme variants displayed kinetic parameters very similar to those of the wild-type enzyme. However, S560A MtIPMS displayed no detectable activity with two different preparations of the enzyme. The results for S560A MtIPMS are in contrast to those for the S560W substitution, which does not affect the activity of the enzyme.

The inhibition parameters for L-leucine were measured for each of the enzyme variants. Briefly, all assay components, including varying amounts of L-leucine, were incubated in a cuvette. Each reaction was initiated by the addition of enzyme. As previously described,[16] values for K_i and K_i* were determined by plotting the L-leucine concentration versus the initial and steady-state velocities, respectively. The K_i value corresponds to the inhibition constant for the initial rapid equilibrium binding, while the K_i* value is the overall inhibition constant for both the fast and slow steps. In the presence of L-leucine, only D563A and H540A MtIPMS display slow-onset inhibition. The remaining enzyme variants display linear progress curves in the presence of L-leucine (Figure 2C and Figure S2 of the Supporting Information) with K_i values ranging from 0.05 to 150 μM (Table 1).

Table 1. L-Leucine Inhibition Constants for Enzyme Variants[a]

enzyme	K_i (μM)	K_i* (μM)
wild type	12 ± 3	2.3 ± 0.2
H540A	23 ± 2	9 ± 1
M559A	155 ± 19	*b*
S560W	51 ± 7	*b*
D563A	3 ± 1	0.05 ± 0.02
D563N	2.3 ± 0.2	*b*
D564A	24 ± 3	*b*
Q566A	7.7 ± 1.6	*b*

[a]The values for K_i and K_i* were determined by replots of the initial and steady-state velocities of progress curves at various concentrations of L-leucine (see the Supporting Information). [b]Enzyme variants displaying only a K_i value displayed linear inhibition kinetics.

On the basis of the thermodynamic model for slow-onset inhibitors, the loss of the slow-onset phenomenon can most simply be described by two possibilities (Scheme 1). First, the height of the barrier between the E−Leu and E*−Leu complexes may have increased such that the E*−Leu complex is no longer kinetically accessible. Second, the height of the barrier could be reduced such that the E*−Leu complex is formed rapidly from the E−Leu complex. Using this model to

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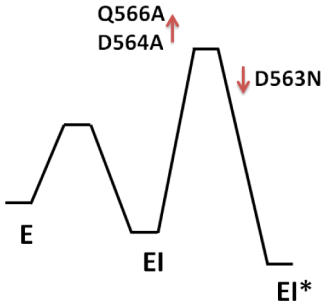
Scheme 1



interpret the effect of loop substitutions, D564A and Q566A can be characterized as increasing the height of the barrier for equilibration as both of these substitutions result in enzymes with K_i values similar to the K_i value determined with the wild-type enzyme. The loss of the slow-onset step in the D563N variant would be interpreted as a decrease in the height of the barrier as the inhibition constant for L-leucine is identical to the K_i* value determined for the wild-type enzyme. The M559A, S560W, and D563A substitutions are more difficult to interpret as these changes have also altered the relative stabilities of the E−Leu complexes.

Overall, the data are consistent with a description of the EI complex as the loop-open enzyme−leucine complex and the EI* complex as the loop-closed enzyme−leucine complex. It is possible that this mechanism exists in most if not all lid-gated effector binding sites; however, it is rarely seen experimentally because of the relationship between the barrier height for loop closure and catalysis. Previous isothermal titration calorimetry results indicate that the free energy change for L-leucine binding is entropy-dominated.[16] This suggests that the thermodynamic driving force for this closure is from the exclusion of water molecules around the hydrophobic portions of L-leucine.

This mechanism is particularly attractive in terms of the variability it affords the enzyme in tailoring the inhibition constant for a ligand. While the binding of L-leucine (or any of the tested amino acids) is sufficient for the inhibition of MtIPMS, the stability of the closed complex dictates the overall inhibition constant. This is supported by the result that loop variants display inhibition constants for L-leucine that range over 3 orders of magnitude around the value determined for the wild-type enzyme without significantly affecting the catalytic parameters.

A precedent for this type of modular mechanism is seen in the allosteric regulation of monomeric human glucokinase. In the presence of glucose, this enzyme is proposed to undergo a slow conformational change to a form with a higher affinity for glucose, resulting in physiologically relevant positive cooperativity.[17] Structurally, this is accomplished by a change in a specific α-helix. Changes in the sequence of the helix alter the cooperative response without drastically affecting the catalytic parameters of the enzyme.[18] A flexible loop has also been implicated in the slow-onset activation of yeast pyruvate decarboxylate by pyruvate.[19] However, in this case the loop appears also to play a role in catalysis.

Allosteric mechanisms that separate the determinants of catalysis, regulation, and effector sensitivity into areas capable of individual control are particularly attractive in terms of enzyme engineering. Our results suggest that targeting residues on an effector binding site loop for mutagenesis may provide a facile route to engineered enzymes with weakened allosteric regulation while retaining full catalytic activity.

■ ASSOCIATED CONTENT

Supporting Information

Initial velocity plots for alternate amino acid inhibitors, kinetic parameters for enzyme variants, progress curves for enzymes not shown in the text, and plots used to determine inhibition constants for enzyme variants. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: pfrantom@ua.edu. Phone: (205) 348-8349.

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Notes

The authors declare no competing financial interest.

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