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V-Type Allosteric Inhibition Is Described by a Shift in the Rate-Determining Step for α-Isopropylmalate Synthase from *Mycobacterium tuberculosis*

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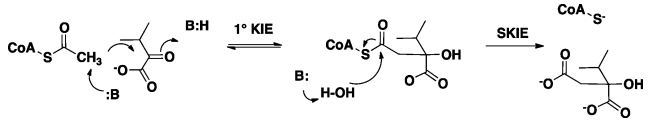
Supporting Information

ABSTRACT: The kinetic parameters affected by allosteric mechanisms contain collections of rate constants that vary based on differences in the relative rates of individual steps in the reaction. Thus, it may not be useful to compare enzymes with similar allosteric mechanisms unless the point of regulation has been identified. Rapid reaction kinetics and kinetic isotope effects provide a detailed description of *V*-type feedback allosteric inhibition in α-isopropylmalate synthase from *Mycobacterium tuberculosis*, an evolutionarily conserved model allosteric system. Results are consistent with a shift in the rate-determining step from product release to the hydrolytic step in catalysis in the presence of the effector.

The identification of evolutionarily conserved structure−function relationships in allosteric mechanisms has proven to be challenging, especially with respect to understanding evolutionary constraints on the acquisition and diversification of allosteric regulation. To accurately identify these relationships, it is essential that the true "function"' of an allosteric mechanism be well understood. Current descriptions of allostery based on changes to the macroscopic kinetic parameters K_M and V_{max}, so-called *K*- and *V*-type mechanisms,[1] respectively, are inadequate for fully describing the functional mechanism of regulation. The values for these kinetic parameters are defined by collections of rate constants for multiple steps in the enzyme reaction and are dependent on the identity of the rate-determining step in the reaction.[2] Thus, similar phenomenological allosteric mechanisms may result from differing physical mechanisms complicating the identification of conserved structure−function relationships. Here the underlying mechanism of *V*-type regulation of the enzyme isopropylmalate synthase from *Mycobacterium tuberculosis* (MtIPMS) by L-leucine is identified. The identification of a discrete mechanism of regulation places MtIPMS in a small group of enzymes characterized in this manner[3,4] and provides a starting place for exploring the diversity of *V*-type allosteric mechanisms.

MtIPMS catalyzes the first step in the biosynthesis of L-leucine and is subject to allosteric feedback inhibition by the amino acid. The enzyme catalyzes a divalent metal-dependent Claisen-like condensation between acetyl-coenzyme A (AcCoA) and ketoisovalerate (KIV) to form an isopropylmalyl-CoA intermediate. The intermediate is hydrolyzed to give the final products isopropylmalate and CoA (Scheme 1).

Scheme 1



Structurally, MtIPMS is a dimeric enzyme with an N-terminal TIM barrel catalytic domain and a conserved C-terminal regulatory domain.[5] In MtIPMS, two molecules of L-leucine act as *V*-type allosteric inhibitors, binding at the interface of the regulatory domain in a noncooperative manner. Multiple experiments have shown that binding of L-leucine does not disrupt the quaternary structure of the enzyme.[6,7] Consistent with the allosteric nature of inhibition, MtIPMS retains approximately 10% activity in the presence of saturating concentrations of L-leucine.

In the absence of L-leucine, product release was proposed to be the rate-determining step in the reaction because of the lack of significant primary and solvent kinetic isotope effects.[7] To confirm this hypothesis, rapid reaction kinetics were used to investigate the first few turnovers of MtIPMS (Figure 1). A burst of product formation is seen followed by a linear progress curve. Dynafit (Biokin, Ltd.)[8] was used to fit the data to the reaction shown in Scheme S1 of the Supporting Information. A burst rate of 17 ± 3 s^{-1} and a steady-state rate of 2.4 ± 0.1 s^{-1} were determined. In the presence of 200 μM L-leucine, a linear progress curve is seen with a rate of 0.4 s^{-1}. Both steady-state rates determined by chemical quench agree with those determined by steady-state kinetics.

The loss of the burst phase in the presence of L-leucine suggests that the rate-determining step in the reaction has shifted from product release to a chemical step. As shown in Scheme 1, MtIPMS has two distinct chemical steps. The rate-determining nature of each step in the presence and absence of L-leucine can be probed by measuring kinetic isotope effects on k_{cat}. Previous results suggest the condensation step is concerted,[7] allowing it to be probed by measuring a primary deuterium isotope effect using $^2H_3$C-AcCoA. Solvent kinetic isotope effects (SKIEs) were determined to probe the hydrolysis step of the reaction. Values for the various kinetic isotope effects are listed in Table 1.

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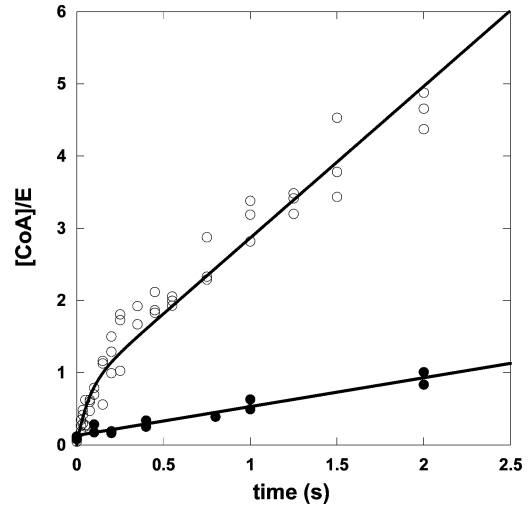

Figure 1. Quench-flow kinetics of MtIPMS in the absence (○) and presence (●) of L-leucine. Solid lines are from a fit of the data to Scheme S1 of the Supporting Information by Dynafit (○) or a linear equation (●).

Table 1. Kinetic Isotope Effects in the Absence and Presence of L-Leucine[a]

	$^{D_2O}k_{cat}$		$^{D}k_{cat}$	
MtIPMS	without leucine	with leucine	without leucine	with leucine
wild type	1.1 ± 0.2	3.3 ± 0.2	1.1 ± 0.1	1.0 ± 0.1
Y410F	1.7 ± 0.3	1.8 ± 0.1	1.2 ± 0.1	1.2 ± 0.1
Q84A	1.2 ± 0.2	2.0 ± 0.1	1.4 ± 0.1	1.2 ± 0.1

[a]Experimental methods and data analysis described in the Supporting Information.

For wild-type (WT) MtIPMS, the presence of 500 μM L-leucine increases the SKIE value from 1.1 to 3.3, suggesting that hydrolysis has become rate-determining in the E−Leu complex (Figure 2). In contrast, the primary deuterium kinetic isotope effect determined with $^{2}H_3C$-AcCoA as a substrate remains unity in the presence of L-leucine. The magnitude of the solvent kinetic isotope effect is larger in magnitude than that reported for MtIPMS when pyruvate is used as an alternate substrate[7] (2.0 ± 0.2) and for malate synthase from *M. tuberculosis* (1.7 ± 0.1), an enzyme that catalyzes the metal-dependent Claisen condensation of glyoxylate and AcCoA.[9] Solvent viscosity effects on k_{cat} values using sucrose as a microviscogen indicate the viscosity of D_2O plays a minor role in the SKIE (Figure S1 of the Supporting Information)

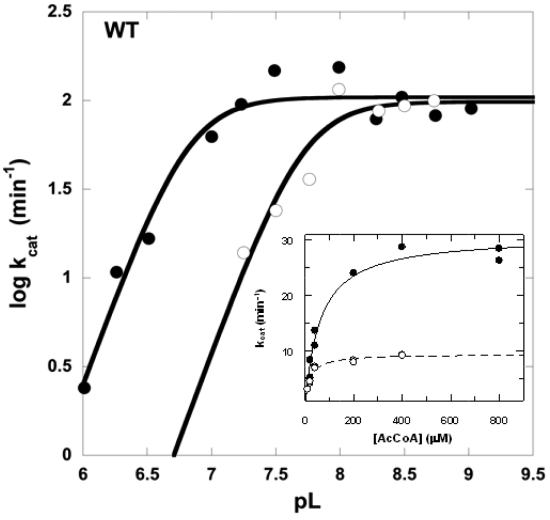

Figure 2. Dependence of k_{cat} values on pL WT MtIPMS in (●) H_2O and (○) D_2O. Solid lines are from a fit to eq S1 of the Supporting Information. The inset shows the SKIE on MtIPMS in the presence of L-leucine at pL 8.5 in (●) H_2O and (○) D_2O. The lines are from a global fit of the data to eq S2 of the Supporting Information.

Several mechanisms could account for a decrease in the rate of hydrolysis caused by L-leucine binding. One possibility is that the affinity of the enzyme for either the necessary divalent or stimulatory monovalent cation has increased. However, titrations of $MgCl_2$ and KCl in the presence of L-leucine indicate no changes in the K_{act} parameter for either metal in the Leu-bound enzyme (Figure S2 of the Supporting Information). Additionally, one could hypothesize that the residual activity of the inhibited enzyme is due to uncoupling of the condensation and hydrolytic steps such that the enzyme is simply hydrolyzing AcCoA to CoA, an event the thiol-capture assay and high-performance liquid chromatography-based assay would not identify. However, nuclear magnetic resonance (NMR) spectroscopy gave no evidence of the formation of acetate by the Leu-bound enzyme, suggesting the reaction is still fully coupled in the presence of L-leucine (Figure S3 of the Supporting Information). Thus, the most likely explanation for the shift in the rate-determining step is that binding of L-leucine perturbs the catalytic machinery used for hydrolysis.

One prediction for an allosteric mechanism based on changes to a discrete step in catalysis is that enzyme variants disrupting the target chemical step may prove to be resistant to inhibition. Two variants of MtIPMS (Y410F and Q84A) exhibit k_{cat} values similar to that of the inhibited WT enzyme (Table S1 of the Supporting Information). Neither enzyme variant displays burst kinetics, suggesting that product release is not rate-determining for either enzyme (Figure S4 of the Supporting Information). Y410F MtIPMS has been previously characterized as feedback resistant with respect to L-leucine.[10] Solution-phase backbone amide H−D exchange experiments suggest this is due to the substitution mimicking the L-leucine-bound form of the enzyme.[6] Thus, one would predict that hydrolysis in Y410F MtIPMS would be rate-determining in the absence of L-leucine. Results shown in Table 1 and Figure S5 of the Supporting Information support this hypothesis as the Y410F variant exhibits significant SKIEs in the absence and presence of L-leucine. In contrast, Q84A MtIPMS is fully responsive to L-leucine despite the substitution's perturbation of catalysis. Binding of L-leucine to the Q84A variant decreases the value of k_{cat} an additional 90%, suggesting that the substitution has perturbed a different step in the mechanism. Indeed, the SKIEs determined with Q84A MtIPMS in the absence and presence of L-leucine exhibit a trend similar to the trend of those determined for the WT enzyme.

Two previous reports investigating the rapid reaction kinetics of enzymes subject to *V*-type allostery concluded that inhibition was accomplished by a shift in the population of active enzyme species due to a conformational change rather than changes to a specific reaction step.[11,12] The rapid-quench results are not sensitive enough to detect a burst of 0.1 equiv of enzyme (the amount consistent with residual activity); thus, this possibility cannot be explicitly ruled out at this time. However, H−D exchange experiments with MtIPMS in the presence of L-leucine exhibit a single isotopic envelope for deuterium

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incorporation.[6] If MtIPMS exhibited multiple populations, multiple deuterium incorporation profiles would be expected for some peptides. What can be concluded from the isotope effect data is that regardless of the population distribution, the residual enzyme activity occurs through an altered reaction diagram with respect to the uninhibited enzyme.

With a detailed description of the *V*-type allosteric mechanism for MtIPMS in hand, it is interesting to speculate about the conservation of the mechanism. Despite the conservation of structure, ligand, and domain organization, enzymes with the LeuA dimer regulatory domain exhibit both *V*-type and *K*-type allosteric regulation.[13] One can propose two possible hypotheses for the diversity in regulation exhibited by a single regulatory domain. One possibility is that following a gene-splicing event, a regulatory domain will evolve specific interactions with a catalytic domain dictating the allosteric mechanism as predicted by colocalization theory.[14] An alternate possibility is that the conserved regulatory domain exerts similar effects on all of the catalytic domains; however, the physical result of that effect with respect to the allosteric mechanism is dependent upon the kinetic mechanism and relative rate constants for each individual catalytic domain. Further biochemical and bioinformatics studies aimed at addressing these issues are underway.

■ ASSOCIATED CONTENT

Supporting Information

Experimental methods, solvent viscosity and cation activity plots, and NMR spectra for product identity. 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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