

Kinetic Evidence for Interdomain Communication in the Allosteric Regulation of α -Isopropylmalate Synthase from *Mycobacterium tuberculosis*[†]

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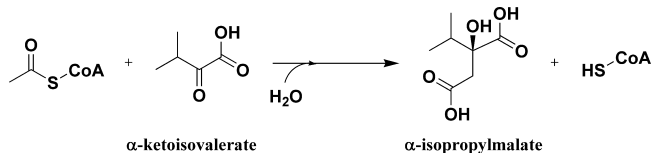
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ABSTRACT: The enzyme α -isopropylmalate synthase from *Mycobacterium tuberculosis* (*Mt*IPMS) has been identified as a possible target for the design of new antitubercular therapeutics. Recently, it was shown that *Mt*IPMS is subject to slow-onset, feedback inhibition by L-leucine, the first instance of an allosteric regulator utilizing this mechanism. Structural studies are inconsistent with canonical allosteric mechanisms, including changes to the quaternary structure or large, rigid-body conformational changes to the enzyme upon L-leucine binding. Thus, the allosteric regulation may result from a discrete inhibitory signal transmitted to the active site upon L-leucine binding in the regulatory domain, a distance of more than 50 Å. To test this mechanism, site-directed mutagenesis was employed to construct enzymes with substitutions at phylogenetically conserved active site residues near the interface of the catalytic and linker domains. The substitutions had wide-ranging effects on the kinetics of L-leucine inhibition, with some modest effects on the kinetic parameters of catalysis. The most dramatic result was the finding that the Y410F mutant form of *Mt*IPMS is insensitive to L-leucine inhibition, suggesting that this residue has completely uncoupled the inhibitory signal to the active site. Overall, the data are consistent with a mechanism of allosteric regulation described by the interdomain communication of the inhibitory signal from the regulatory to catalytic domain and implicate the interactions between the linker and catalytic domains as critical determinants of inhibitory signal transmission.

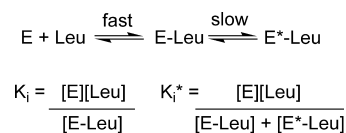
Understanding the mechanism of allosteric regulation in proteins, where an effector molecule exerts influence at a distal site, has been a central pursuit of biochemistry for more than 40 years (1). Cases in which the binding of an effector molecule causes a change in the quaternary structure of a protein or alters the equilibrium between active and inactive conformers through rigid-body motion have been well documented due to their distinct structural and physical properties (2, 3). However, there are numerous reports of proteins that are allosterically regulated which are not consistent with either of these two models (4–7). In these cases, the mechanism of allostery is loosely described by invoking changes in local protein dynamics or equilibria transmitted through a type of contact network between the binding site of the effector molecule and the active site.

A nearly ubiquitous type of allosteric regulation is described as feedback inhibition. This type of inhibition refers to the regulation of enzymes in biosynthetic pathways by

Scheme 1



Scheme 2



the downstream end product of the pathway. Recently, we described the feedback inhibition of the enzyme α -isopropylmalate synthase from *Mycobacterium tuberculosis* (*Mt*IPMS)¹ by L-leucine (8). IPMS catalyzes the acetyl-CoA-dependent carboxymethylation of α -ketoisovalerate (α -KIV) to form α -isopropylmalate (Scheme 1). This reaction is the first committed step in the biosynthesis of L-leucine and as such is subject to feedback inhibition (9). However, closer inspection of the initial velocity progress curves revealed biphasic kinetics. Further kinetic studies established that for the *M. tuberculosis* enzyme L-leucine acts as a reversible, slow-onset inhibitor (a two-step process in which the initial enzyme–leucine complex undergoes a slow equilibration

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¹ Abbreviations: *Mt*IPMS, *M. tuberculosis* α -isopropylmalate synthase; α -KIV, α -ketoisovalerate; AcCoA, acetylcoenzyme A; α -IPM, α -isopropylmalate; DTP, 4,4'-dithiodipyridine.

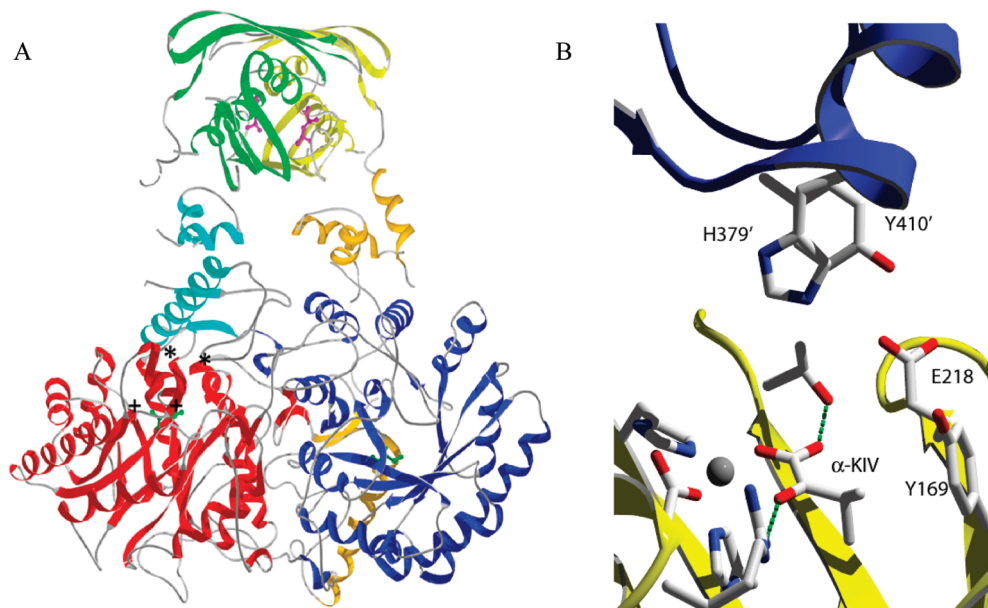


FIGURE 1: Ribbon drawing of the three-dimensional structure of α -isopropylmalate synthase from *M. tuberculosis*. (A) Domains colored red and blue, orange and cyan, and yellow and green correspond to the catalytic, linking, and regulatory domains, respectively, of each monomer. L-Leucine is colored purple in the regulatory domain. The substrate (α -KIV) is colored green to denote the active site. The general locations of substituted residues in this study are denoted with asterisks for residues in the linker domain and with pluses for residues in the catalytic domain. (B) Detailed rendering of residues on monomer A (yellow) and monomer B (purple and prime numbering) selected for mutational analysis. Residues involved in metal binding and α -KIV binding are shown as well. All three-dimensional structures were created with SwissPDB Viewer and rendered with POVray 3.5.

with a more inhibited form). To the best of our knowledge, this is the first reported case of an allosteric inhibitor acting through a slow-onset mechanism for any enzyme system.

The slow-onset inhibition by L-leucine was consistent with the mechanism shown in Scheme 2, a minimal kinetic mechanism without regard to substrate binding (10). Binding of L-leucine to the free enzyme rapidly forms the inhibited binary complex. The binary complex then undergoes a slow equilibration to a second form of the enzyme which displays a further decrease in activity. The characterization of L-leucine as a slow-onset inhibitor is unique in the fact that all other reported slow-onset inhibitors have been characterized as competitive inhibitors (i.e., at saturating concentrations of substrate, inhibition is relieved), while kinetic studies with L-leucine are consistent with noncompetitive inhibition versus α -KIV. Slow-onset inhibition is traditionally exhibited by transition state or bisubstrate analogue inhibitors. The mechanism describing slow-onset inhibition by these types of inhibitors is based on the conversion of the transition state stabilization energy into binding energy in the conversion from the EI to EI* complex. In the case of a slow-onset allosteric inhibitor, it is difficult to rationalize the involvement of the transition state stabilization energy in the mechanism of inhibition. As this type of inhibition is particularly useful in developing therapeutics due to the dramatic increase in the residence time of the inhibitor, it is important to develop a better understanding of the mechanism of slow-onset inhibition in an allosteric background.

The three-dimensional structure of the *Mt*IPMS- α KIV- M^{2+} ternary complex has been determined in the presence and absence of L-leucine (11). The enzyme is a homodimer with each monomer consisting of three domains: an N-terminal TIM-barrel catalytic domain (residues 51–368), a subdivided linker domain (residues 369–424 and 434–490), and a C-terminal regulatory domain (residues 491–644)

(Figure 1A). L-Leucine can be found bound to the regulatory domain (one molecule per monomer) some 50 Å from the active site. However, a comparison of the crystallographically determined structures in the absence and presence of L-leucine shows virtually no changes in the architecture of the active site. Additionally, studies have shown no effect of L-leucine binding on the quaternary structure of *Mt*IPMS, ruling out a mechanism of allosteric regulation involving changes to the quaternary structure of the enzyme (12). Thus, in the absence of structural evidence consistent with a large, rigid-body structural rearrangement or changes to the quaternary structure upon L-leucine binding, a mechanism involving interdomain communication between the regulatory domain and the active site seems like the most plausible mechanism for describing the allosteric inhibition.

To test this theory, we have constructed a selected number of mutant forms of *Mt*IPMS in the region of the protein where linker subdomain I (residues 369–424) interacts with the active site (Figure 1B). In the *Mt*IPMS dimer, each monomer forms a distinct catalytic domain, but the polypeptide chains then extend such that linker subdomain I of one chain interacts with the active site of the adjacent monomer before forming the remaining portions of the linker and regulatory domains. On the basis of a sequence alignment, this region contains several phylogenetically conserved residues which we hypothesize may be important for allosteric control. Here, we describe the effects of substitutions at these conserved residues in *Mt*IPMS on the kinetics of the enzyme reaction and inhibition by L-leucine.

MATERIALS AND METHODS

Materials. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs, and *Pfu* turbo DNA polymerase was from Stratagene. Oligonucleotides used to

amplify the *leuA* gene were obtained from Invitrogen. All substrates, buffers, and chemicals were obtained from Sigma and Aldrich. The Zero-Blunt Kit was obtained from Invitrogen. Plasmid pET28a(+) and *Escherichia coli* BL21(DE3)pLysS cells were from Novagen. Chromatographic columns and resins were obtained from Amersham-Pharmacia Biotech, and Ni-NTA resin was from Novagen.

Cloning and Expression of Mutant IPMS. Point mutations in the pET28a(+):*leuA* plasmid were introduced using QuikChange site-directed mutagenesis kit (Stratagene). The integrity of the gene and the presence of the desired point mutations were confirmed by DNA sequencing. The mutant plasmids were transformed into BL21(DE3)pLysS cells. BL21(DE3)pLysS cells harboring pET28a(+):*leuA* and variants were inoculated into 2× LB medium, grown at 37 °C until they reached an OD₆₀₀ of 0.4, induced with 1 mM IPTG, and incubated at 25 °C for 4 h. Protein expression and purity were analyzed by SDS–PAGE.

Protein Purification. All steps were performed at 4 °C. Cells were thawed on ice for 30 min with Complete protease inhibitors (Roche), lysozyme, and DNase I in 20 mM TEA (pH 7.8) containing 300 mM NaCl and 50 mM imidazole. Cells were lysed by sonication, and the soluble extract was recovered after two centrifugations at 20000 rpm. The clear soluble fraction was loaded onto a nickel-NTA column (100 mL) and eluted with a linear 50 to 500 mM imidazole gradient in 20 mM TEA (pH 7.8) containing 300 mM NaCl. The peak fractions were pooled and dialyzed against 10 L of 20 mM TEA (pH 7.8). The dialysate was loaded onto a Mono-Q column and eluted with a linear 0 to 500 mM NaCl gradient in 20 mM TEA (pH 7.8). The peak fractions were pooled and dialyzed against 10 L of 20 mM TEA (pH 7.8). The dialysate was made 1 M in (NH₄)₂SO₄, loaded onto a Phenyl Sepharose column, and eluted with a linear 1 to 0 M (NH₄)₂SO₄ gradient in 20 mM TEA (pH 7.8). Fractions containing pure IPMS were pooled and concentrated. After concentration, the IPMS solution was dialyzed against 10 L of 20 mM TEA (pH 7.8) containing 2 mM EDTA and then dialyzed versus 10 L of 20 mM TEA (pH 7.8). The protein was stored at –20 °C in 50% glycerol.

Measurement of Enzymatic Activity. Initial velocities for the forward reaction of α-isopropylmalate synthase were determined using 4,4'-dithiodipyridine (DTP) to detect the formation of CoA at 324 nm ($\epsilon = 19800 \text{ M}^{-1} \text{ cm}^{-1}$) at 25 °C. A typical reaction mix contained 50 mM potassium phosphate (pH 7.5), 12 mM MgCl₂, 50 μM DTP, 300 μM acetyl-CoA (AcCoA), and 300 μM α-ketoisovalerate (α-KIV). Values for the kinetic constants were determined using either the Michaelis–Menten equation (eq 1) or, in enzymes exhibiting substrate activation, eq 2, where V_1 and V_2 are the maximal velocities and K_1 and K_2 are the Michaelis constants for each phase of the kinetics, respectively.

$$\frac{v}{E_{\text{tot}}} = \frac{k_{\text{cat}}[S]}{K_m + [S]} \quad (1)$$

$$\frac{v}{E_{\text{tot}}} = \frac{V_1[S]}{K_1 + [S]} + \frac{V_2[S]}{K_2 + [S]} \quad (2)$$

Inhibition by L-Leucine. For inhibition assays, reaction mixtures contained 50 mM potassium phosphate buffer (pH 7.5), 12 mM MgCl₂, 100 μM DTP, 300 μM AcCoA, 300

μM α-KIV, and variable concentrations of L-leucine. Reactions were initiated by the addition of *Mt*IPMS.

Data Analysis. Progress curves at various L-leucine concentrations were fit to eq 3, where $[P]_t$ is the total product produced, $[E]_t$ is the total enzyme, v_{ss} is the steady state velocity, t is time, v_i is the initial velocity, k_b is the exponential rate constant for equilibration, and C is a finite intercept (10). The C term is included to allow for any product displacement at time zero since a minimal mixing time of 2 s is required prior to measurement (10). The terms v_i and v_{ss} were then plotted versus L-leucine concentration. The dependence of v_i and v_{ss} on L-leucine concentration was then analyzed as described below.

$$\frac{[P]_t}{[E]_t} = v_{ss}t + \frac{(v_i - v_{ss})(1 - e^{-k_bt})}{k_b} + C \quad (3)$$

Theory. The feedback inhibition of *Mt*IPMS by L-leucine is accurately defined as noncooperative allosteric inhibition; in situations where the binding of inhibitor and binding of substrate are independent of each other, this mechanism is identical to noncompetitive inhibition (13).² The velocity equation for this type of allosteric mechanism is shown in eq 4, where V is the velocity in the absence of inhibitor, K_i is the dissociation constant for the inhibitor, β is the fractional activity relative to V at saturating inhibitor concentrations, and K_s is the dissociation constant for the substrate. Under saturating substrate conditions, this equation can be reduced to eq 5. The initial velocities determined using eq 1 were plotted versus L-leucine concentration and fit to eq 5 to determine values for V , β , and K_i .

$$\frac{v}{E_T} = \frac{V[S]K_i + \beta V[S][I]}{[S]K_i + [I]K_s + [S][I] + K_iK_s} \quad (4)$$

$$\frac{v}{E_T} = \frac{VK_i + \beta V[I]}{[I] + K_i} \quad (5)$$

In the cases of complete inhibition, plots of initial velocities versus L-leucine concentration were fit by eq 5 with a β of 0. By analogy with the many descriptions of competitive slow-onset inhibitors, when the steady state velocities are used in place of the initial velocities, the K_i term is replaced with the term K_i^* , which describes the overall dissociation constant for dissociation of L-leucine from the enzyme (10). In the simplest case, the values for the other terms in the equation should be identical regardless of whether the initial or steady state velocities are analyzed.

Sedimentation Velocity Experiments. Sedimentation velocity measurements were conducted at 30000 rpm in a Beckman XL-I analytical ultracentrifuge using double-sector cells and the Ti-60 rotor at 22 °C in pH 7.5 buffer containing 20 mM potassium phosphate and 20 mM MgCl₂. Boundary movement was followed at 280 nm using the centrifuge's absorption optics. Each sample was adjusted to $A_{280} = 1.0$ data to facilitate their direct comparison. $g(s^*)$ distributions were determined using DCDT+ version 2.0.9 (14). A \bar{v} value of 0.7276 was calculated from the amino acid composition using Sednterp version 1.06 (15), as were the density

² An alternate kinetic analysis of allosteric effectors has previously been discussed (26). In the absence of cooperativity, the equations in this reference are essentially identical to those presented by Segel (13).

Table 1: Steady State Kinetic Parameters for *MtIPMS* and Mutant Enzymes^a

enzyme	α -KIV ^b				acetyl-CoA ^b			
	k_{cat1} (min ⁻¹)	K_1 (μ M)	k_{cat2} (min ⁻¹)	K_2 (μ M)	k_{cat1} (min ⁻¹)	K_1 (μ M)	k_{cat2} (min ⁻¹)	K_2 (μ M)
WT	152 \pm 3	7.1 \pm 0.5	—	—	163 \pm 3	24 \pm 2	—	—
Y169F	285 \pm 16	13.4 \pm 2.6	—	—	340 \pm 13	40 \pm 6	—	—
E218A	2.2 \pm 0.1	1.6 \pm 0.2	1.3 \pm 0.1	39 \pm 11	3.5 \pm 0.2	9.5 \pm 1.1	5.6 \pm 2.8	697 \pm 615
H379A	42 \pm 18	1.6 \pm 1.1	89 \pm 17	19 \pm 6	153 \pm 2	23 \pm 1.5	—	—
Y410F	4.5 \pm 0.2	0.5 \pm 0.1	—	—	5.6 \pm 0.2	1.1 \pm 0.2	—	—

^a All reactions were performed as described in Materials and Methods. ^b Varied substrate. ^c For enzymes displaying substrate activation, kinetic parameters with subscripts 1 and 2 represent the kinetic parameters for the unactivated and activated forms of the enzyme, respectively. For enzymes without substrate activation, k_{cat1} and K_1 are k_{cat} and K_m , respectively.

(1.01356 g/mL) and viscosity (1.0445 cp) from the buffer composition. The latter values were used to correct the sedimentation coefficients to $S_{20,w}$.

Isothermal Titration Calorimetry Experiments. Calorimetric titrations of wild-type (WT), Y410F, and E218A IPMS with L-leucine were performed on a VP-ITC microcalorimeter (MicroCal). Protein samples were dialyzed against 50 mM Tris, 25 mM KCl, and 12 mM MgCl₂ (pH 7.5). The ligand solution was prepared by dissolving the sample in protein dialysate buffer, and both ligand and protein solutions were degassed for 5 min under vacuum prior to each titration. The concentration of protein was determined using Bradford reagent, and the concentration of L-leucine was determined by weight. The 1.46 mL sample cell was filled with a 100 μ M protein solution and the 250 μ L injection syringe with a 1 mM L-leucine solution which was injected at a rate of 10 μ L every 4 min into the sample cell. The titrations were performed at 25 °C, and the data were fit using the One Set of Sites Model provided in Origin 7.0 to determine K_a , ΔH , and N (the stoichiometry of binding).

Product Analysis by ¹H NMR. The reaction of Y410F IPMS with AcCoA and α -KIV was followed by ¹H NMR. The reaction mixture consisted of 50 mM potassium phosphate (pH 7.5), 12 mM MgCl₂, 1.1 mM α -KIV, 1 mM AcCoA, and 100 μ g of Y410F IPMS. After enzyme addition, the reaction was allowed to proceed at room temperature for 16 h.

RESULTS

Multiple-Sequence Alignment. Multiple-sequence alignment of 12 α -IPMS's from eubacteria, archaea, plants, and fungi was performed using ClustalW (Figure S1 of the Supporting Information) (16). The overall level of identity is 5.6% (equivalent to 35 amino acids), with an additional 4.6% of residues showing a strong degree of similarity. The majority of conserved residues map to the active site and subdomain I of the linker domain. Four residues (three strictly conserved, one strongly similar) that reside at the interface of the catalytic domain and linker subdomain I of the adjacent monomer were selected for mutational analysis (Figure 1). Residues Y169 and Y410 were mutated to phenylalanine, and residues E218 and H379 were mutated to alanine.

Steady State Kinetic Analysis. The kinetic parameters determined for the mutant enzymes are listed in Table 1. Two of the mutant enzymes, E218A and Y410F IPMS, exhibit k_{cat} values that are reduced 35–40-fold relative to that of wild-type *MtIPMS*. If Y410F IPMS is excluded, none of the mutations have caused a drastic change in the K_m values for either substrate. In the case of Y410F IPMS, the

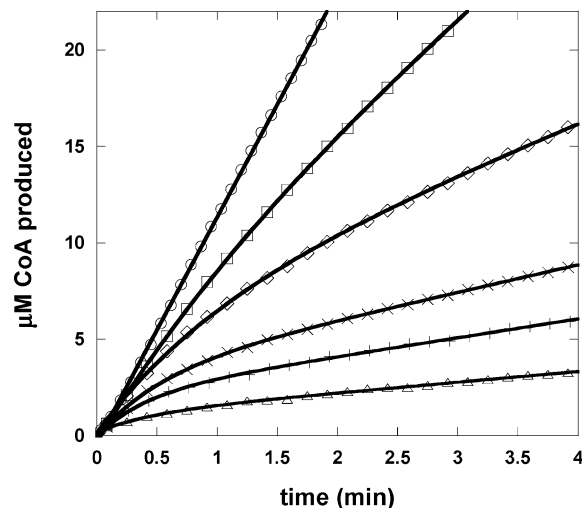


FIGURE 2: Kinetics of slow-onset inhibition of Y169F *MtIPMS* by L-leucine. Reactions were initiated with enzyme and the following concentrations of L-leucine: 0 (\circ), 2.5 (\square), 5 (\diamond), 10 (\times), 15 ($+$), and 50 μ M (\triangle). Solid black lines are fits to either a linear equation (0 μ M leucine) or eq 3.

K_m values are actually lowered \sim 10-fold relative to that of the wild-type enzyme. Two of the substitutions, E218A and H379A, resulted in enzymes which display substrate activation toward α -KIV (Figure S2 of the Supporting Information), suggesting that these mutations have created nonsymmetrical active sites in the dimer. In addition, E218A IPMS also displays substrate activation kinetics toward AcCoA, but with a significantly higher K_m for the second binding site (10 and 700 μ M).

Effect of Mutations on Inhibition by L-Leucine. With the exception of Y410F IPMS, all of the variant enzymes displayed slow-onset inhibition kinetics (i.e., biphasic progress curves) in the presence of L-leucine (Figure 2 for Y169F IPMS). In the absence of L-leucine, all enzymes exhibited linear kinetics over the time frame of the inhibition experiments, suggesting that the biphasic kinetics were not due to enzyme instability or tighter product inhibition (data not shown). Inhibition by L-leucine was characterized by evaluating the initial and steady state velocities of the enzymes at various concentrations of inhibitor as described in Materials and Methods (Figure 3). The inhibition parameters determined for the wild-type and mutant enzymes are listed in Table 2. Closer inspection of the kinetic data for the wild-type enzyme revealed that at saturating concentrations of L-leucine (200 μ M or 17 times the K_i value) the enzyme retained approximately 10% activity. The values determined for K_i and K_i^* for the wild-type enzyme are identical to those determined previously (8). The Y169F substitution resulted in an enzyme that was fully inhibited in the presence of

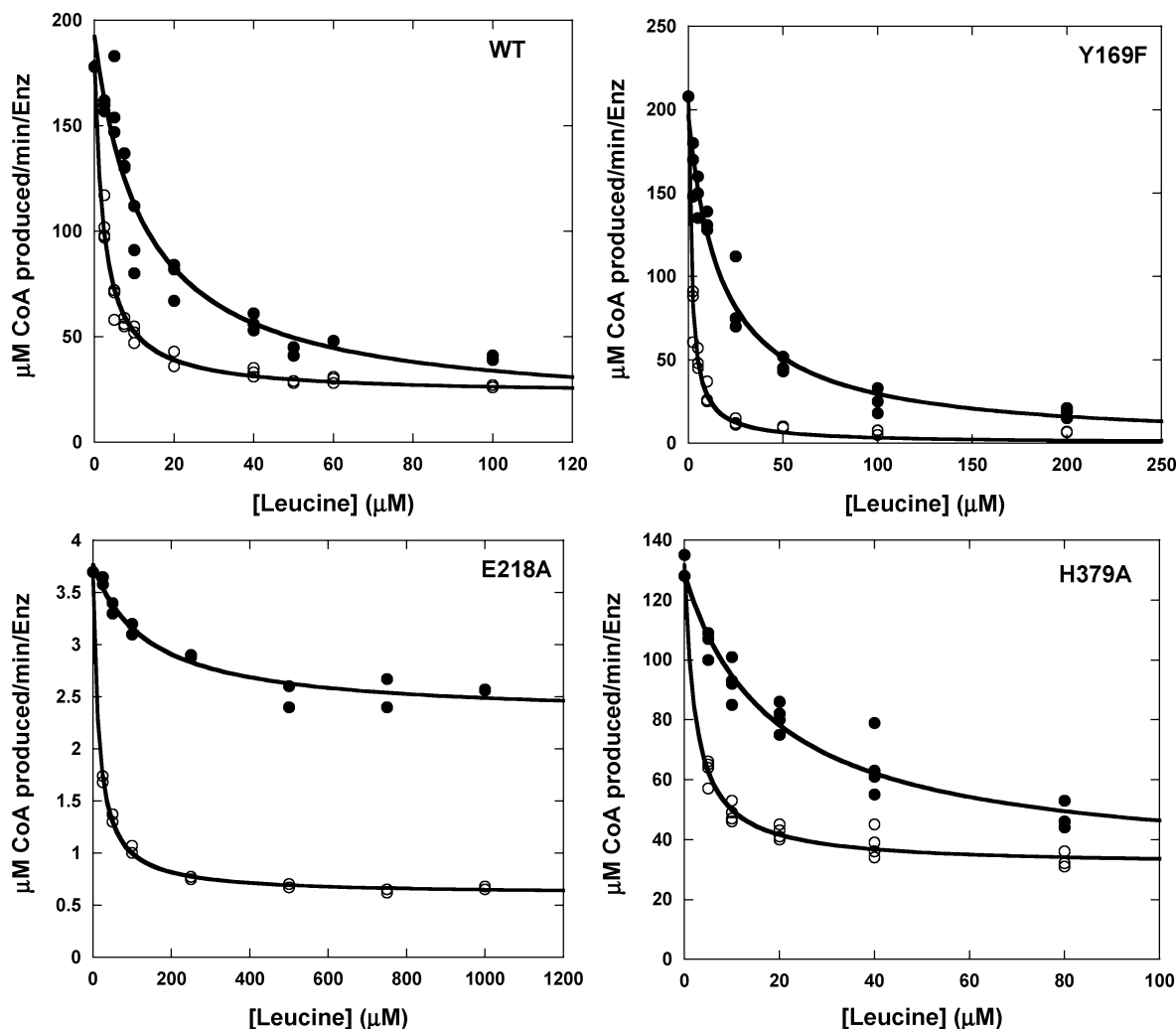


FIGURE 3: Plots of v_i (●) and v_{ss} (○) vs L-leucine concentration for the wild-type, Y169F, E218A, and H379A enzymes. Solid lines are from fits to eq 5. Results are listed in Table 2.

Table 2: L-Leucine Inhibition Parameters for *MtIPMS* and Mutant Enzymes^a

enzyme	K_i (μM)	K_i^* (μM)	β
WT	12 ± 3	2.3 ± 0.2	0.1
Y169F	18 ± 2	1.6 ± 0.1	— ^b
E218A	139 ± 42	15 ± 1	0.61 and 0.16 ^c
H379A	19 ± 4	2.3 ± 0.2	0.24
Y410F	— ^d	—	—

^a Performed as described in Materials and Methods. ^b Data for Y169F IPMS were fit to eq 5 with a β of 0. ^c Limiting values for v_i and v_{ss} , respectively. ^d Inhibition not observed.

L-leucine, while the H379A substitution retained 24% activity, without affecting the K_i or K_i^* values relative to the wild-type values. This suggests that these residues have not perturbed the binding of L-leucine but have altered the environment of the active site in the E^* –leucine complex, resulting in differing residual activities.

Two of the mutant enzymes exhibited much more dramatic alterations in inhibition kinetics. The results from the E218A enzyme suggest that this substitution has caused a change in the affinity of the free enzyme for L-leucine due to the 10-fold increase in the value for K_i (a true dissociation constant) relative to the value for the wild-type enzyme. Since the K_i^* value (in Scheme 2) consists of an arrangement of the rate constants involved in the second step multiplied

by the K_i value, the 10-fold change in K_i could account for the 6.5-fold change in the K_i^* value. E218A IPMS also gave different values for the residual activity depending on the analysis of the v_i or v_{ss} data, retaining 61 and 16% activity, respectively. This result suggests that the E218A substitution has also caused a change in the active site architecture of the E–leucine complex resulting in significantly higher residual activity. The most interesting result was seen for the Y410F substitution. This mutant enzyme was insensitive to L-leucine at concentrations up to 500 μM .

Effect of L-Leucine on Quaternary Structure. It has previously been determined that WT *MtIPMS* is a stable dimer in solution and addition of L-leucine has no effect on the quaternary structure (12). However, it has been reported that the mechanism of allosteric inhibition by L-leucine of α -IPMS from *Salmonella enterica* serovar typhimurium is consistent with the dissociation of a tetramer to inactive monomers (17, 18). To determine if the differences in inhibition kinetics seen with the mutant enzymes were due to a change in quaternary structure, sedimentation velocity experiments were conducted in the presence and absence of 1 mM L-leucine. The sedimentation coefficients determined for each enzyme (except Y169F IPMS) in the absence of L-leucine were consistent with a dimer for all of the enzymes (Table S1 of the Supporting Information). When L-leucine

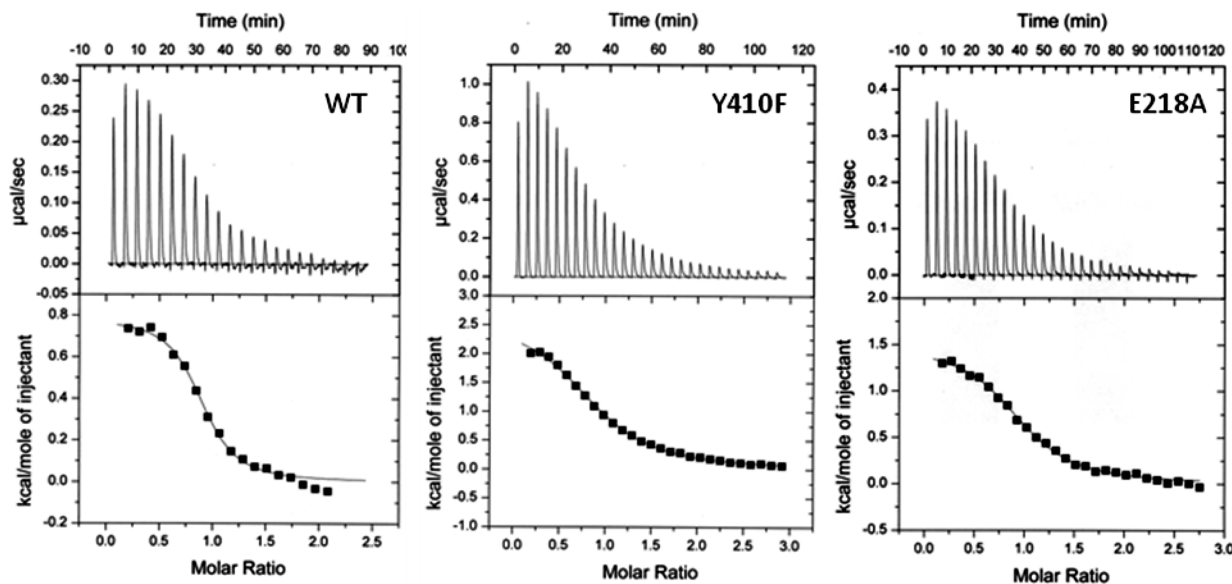


FIGURE 4: Isothermal titration calorimetry plots for wild-type, Y410F, and E218A *Mt*IPMS. Experiments were performed as described in Materials and Methods. A fit of the data to a one-set of sites model produced the following values for the binding of L-leucine to the wild-type ($N = 0.86 \pm 0.02$, $\Delta H = 774 \pm 20$ cal/mol, and $\Delta S = 27.8$ cal mol⁻¹ K⁻¹), Y410F ($N = 0.91 \pm 0.01$, $\Delta H = 2598 \pm 48$ cal/mol, and $\Delta S = 30.1$ cal mol⁻¹ K⁻¹), and E218A ($N = 0.97 \pm 0.01$, $\Delta H = 1486 \pm 31$ cal/mol, and $\Delta S = 28.5$ cal mol⁻¹ K⁻¹) enzymes.

was added, no significant changes were seen in the sedimentation coefficients. This result suggests that the mutations have not altered the quaternary structure of the enzyme and that the altered inhibition kinetics are not due to a dissociation of the active dimer complex in the wild-type or mutant enzymes.

Further Characterization of Y410F IPMS. Because of the unanticipated and dramatic effect of the Y410F substitution, this enzyme form was investigated in greater detail. The spectroscopic assay described for IPMS measures production of CoA. In the wild-type enzyme, the production of CoA has been shown to be strictly coupled to the formation of α -IPM, with no evidence of uncoupled AcCoA hydrolysis (12). To ensure that the activity associated with Y410F IPMS is due to α -IPM production, ¹H NMR was used to monitor the reaction products. There was no evidence of the Y410F-catalyzed hydrolysis of AcCoA in the presence of α -KIV (Figure S3 of the Supporting Information).

To determine if the loss of sensitivity to L-leucine in the Y410F enzyme is due to the inability to bind L-leucine, isothermal titration calorimetry (ITC) was employed to directly determine the dissociation constant for L-leucine for the wild-type and Y410F enzymes (Figure 4). Binding of L-leucine to the wild-type enzyme was endothermic and could be fit to a one-site model consistent with the noncooperative kinetics of L-leucine inhibition. A K_d value of 3.0 ± 0.6 μ M was determined for the wild-type enzyme, consistent with the value for K_i^* . Y410F IPMS gave ITC data which could be fit to the same equation, and a value of 21 ± 1 μ M was determined for the dissociation constant for L-leucine, suggesting that the substitution has not drastically altered the affinity of the enzyme for L-leucine.

DISCUSSION

The enzyme α -IPMS from *M. tuberculosis* catalyzes the first step in the biosynthesis of the amino acid L-leucine. We have previously demonstrated the *Mt*IPMS is feedback inhibited by L-leucine through a slow-onset mechanism (8).

We believe that this is the first reported case of an allosteric effector that acts through a slow-onset mechanism; all previous inhibitors displaying slow-onset kinetics have been characterized as competitive inhibitors. In the wild-type enzyme, there is no evidence of L-leucine causing a large, rigid-body conformational change or altering the quaternary structure of the enzyme, ruling out either of these allosteric mechanisms. Thus, we hypothesized that there must be some type of interdomain communication between the regulatory domain and the active site to achieve inhibition. This type of interdomain communication has also been proposed in the more prevalent ACT regulatory domain-containing enzymes that are responsible for the regulation of a number of metabolic systems involving amino acid metabolism (19). Studies of the enzyme D-3-phosphoglycerate dehydrogenase (PGDH), which catalyzes the first step in the biosynthesis of D-serine, demonstrate that PGDH is subject to feedback regulation by D-serine as a V-type inhibitor similar to that seen in *Mt*IPMS (20). Recently, structures of both the apo and serine-bound enzyme from *M. tuberculosis* have been reported which demonstrate that serine binding produces only subtle movements of residues involved in binding the inhibitor and no domain movement, consistent with an allosteric mechanism involving interdomain communication (21, 22).

According to the three-dimensional structure of *Mt*IPMS, a probable intersection for this type of communication would occur where linker subdomain I of one monomer interacts with the catalytic domain of the adjacent monomer (11). To probe this interface, mutagenesis of specific residues at the interface was undertaken in an attempt to disrupt the allosteric communication. With respect to the roles of the selected residues in catalysis, rather than regulation, an analysis of the kinetics of the substituted enzymes was surprising rather than enlightening. *Mt*IPMS belongs to a family of enzymes that catalyze the Claisen condensation reaction between an α -keto acid and acetyl-CoA. Within this family, the enzyme homocitrate synthase catalyzes the carboxymethylation of α -ketoglutarate by acetyl-CoA. Recently, mutational studies

on homocitrate synthase from *Saccharomyces cerevisiae* have concluded that the residues equivalent to E218 and H379 act as a catalytic dyad in the chemical mechanism (23). In fact, no detectable activity was seen when the histidine residue was mutated to alanine, and the substitution of alanine for the glutamate residue reduced the k_{cat} value 1000-fold, in sharp contrast to the results with *Mt*IPMS. However, the mutation equivalent to Y410F in homocitrate synthase had a modest effect on activity, lowering the k_{cat} value only 25-fold similar to the effect we report. In light of the comparison with homocitrate synthase, it is clear that none of these residues are critical for catalysis, as evidenced by the maximal drop of ~ 40 -fold in the k_{cat} value. In addition, none of the mutations adversely affects the Michaelis constants for either substrate. However, it is known that for wild-type *Mt*IPMS chemistry is not rate-limiting, making interpretation of the Michaelis constant difficult (12). Consistent with a proposed rate-limiting product release, the Y169F substitution results in an enzyme that exhibits a 2-fold increase in the value for k_{cat} relative to the wild-type enzyme. This residue is located between the α -KIV binding site and the opening of the active site cleft, suggesting that loss of the hydroxyl group allows for more facile release of the product from the active site.

Two of the substitutions appear to have created kinetic cooperativity between the two active sites. E218A and H379A IPMS have initial velocity kinetics consistent with substrate activation toward α -KIV. In the three-dimensional structure, these two residues (one from each monomer, thus E218A and H379'A) are located 4.5 Å from each other and within 5–6 Å of α -KIV. The simplest explanation for these results is that removal of these charged residues at the active site interface of the two monomers has resulted in nonsymmetrical active site architecture in the presence of α -KIV. Another possible explanation for substrate activation kinetics which cannot be explicitly ruled out is the presence of a contaminating enzyme. However, neither of these variant enzymes produces CoA in the absence of α -KIV under the experimental conditions, suggesting that any contaminating enzyme must also be dependent on α -KIV, making this explanation unlikely. E218A IPMS also displays substrate activation toward AcCoA; however, this result is less interpretable because of the inability to identify the binding site of AcCoA in structural studies.

The effects of the mutations on L-leucine inhibition of IPMS were determined by analysis of the initial and steady state velocities of the biphasic reaction. Surprisingly, all of the mutant enzymes (except Y410F IPMS) exhibited slow-onset inhibition kinetics. This result suggests that the E–Leu to E*–Leu transition is not governed through this domain interface and may be the result of a more global change in conformation. Initial attempts to fit the initial velocity data for the L-leucine inhibition of the wild-type enzyme to an equation derived for competitive slow-onset inhibitors quickly led to the realization that the wild-type enzyme retains approximately 10% activity at saturating L-leucine concentrations. However, in a slow-onset mechanism where there are two distinct enzyme–inhibitor forms (E–Leu and E*–Leu) it is difficult to determine conclusively whether the residual activity is due to a single form, or a combination of both forms.

The activity of Y169F IPMS, however, is completely inhibited in the presence of saturating concentrations of L-leucine. This substitution has not affected the K_i or K_i^* values for L-leucine, but it has altered the architecture of the active site in the presence of L-leucine relative to the wild-type enzyme. H379A IPMS retains 24% activity in the presence of saturating concentrations of L-leucine, more than twice the relative residual activity of the wild-type enzyme. The results seen with these substitutions demonstrate that changes in the linker domain–active site interface can affect the kinetics of inhibition without altering the binding of L-leucine.

More drastic effects are seen with the E218A enzyme. The inhibition constants for L-leucine determined with E218A IPMS are elevated (6.5–10-fold) relative to the values determined with the wild-type enzyme. This suggests that the substitution has moderately affected the affinity of the enzyme for L-leucine, despite the fact that the residue is 50 Å from the inhibitor binding site. A K_d value of 7.1 ± 0.8 μM for L-leucine binding to the E218A enzyme was determined using ITC experiments (Figure 4). This value is elevated 2.3-fold relative to the K_d value determined with the wild-type enzyme. The E218A substitution has also significantly altered the residual activity of the enzyme–Leu complexes. The initial velocity retains nearly 60% of the uninhibited activity, while the final, steady state velocity only retains 16% activity. This suggests that changes at the linker domain–active site interface can influence the architecture of the L-leucine binding site and the active site when L-leucine is bound.

Kinetic experiments with Y410F IPMS suggest that this substitution has rendered the enzyme insensitive to L-leucine. Control experiments confirmed that Y410F IPMS has not abolished L-leucine binding, is chemically competent, and exists as a stable dimer, ruling out these alternate explanations for the absence of inhibition. The results from ITC experiments are consistent with the Y410F mutation altering the affinity of the enzyme for L-leucine 7-fold, relative to the value determined with the wild-type enzyme. The ability of the Y410F and E218A substitutions to alter the affinity of the enzyme for L-leucine is consistent with the presence of a network of residues connecting the active site and the regulatory domain. In addition, the Y410F substitution appears to have severed the network as evidenced by the uncoupling of the binding of L-leucine in the regulatory domain from changes to the active site architecture.

From the crystal structure, it was straightforward to propose that the Y410-containing loop plays a role in transmitting the signal from linker subdomain I to the active site (11); however, the remainder of the network lacks similar definition. Mutations that resulted in resistance to inhibition by trifluoroleucine produced by genetic studies on IPMS from *Saccharomyces cerevisiae* all mapped to the regulatory domain near the L-leucine binding site (24). Only one of the residues implicated in trifluoroleucine resistance, A568, is positioned at the interface of the regulatory domain and subdomain II of the linker domain. This interface is made up of three β -sheets on the regulatory domain and the three helices of subdomain II of the linker domain. There are numerous possible interactions consisting of ionic and aromatic residues, making identification of important interactions difficult. Tracing the proposed network between the

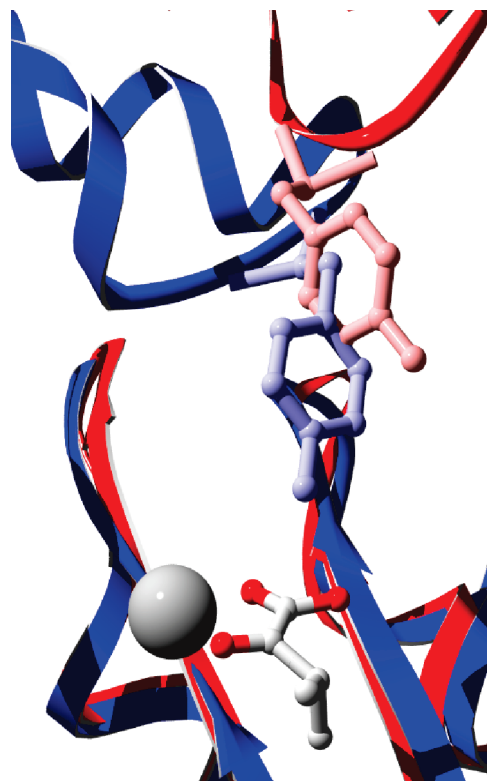


FIGURE 5: Superimposition of *Mt*IPMS and DmpG. Swiss-PDB Viewer was used to align the E chain of DmpG (Protein Data Bank entry 1NMV) (blue) with *Mt*IPMS (Protein Data Bank entry 1SR9) (red). The alignment included 808 atoms and had a root-mean-square deviation of 1.4 Å. α -KIV and a Mg^{2+} atom are shown to indicate the active site of *Mt*IPMS. *Mt*IPMS Y410 (red) and DmpG Y291 (blue) are depicted as ball-and-stick representations.

linker subdomains becomes even more challenging due to asymmetry and missing electron density in the crystal structure.

Additional support for the inclusion of the Y410-containing loop in an interdomain allosteric network comes from the three-dimensional structure of the bifunctional enzyme 4-hydroxy-2-ketovalerate aldolase (DmpG)/acylating acetaldehyde dehydrogenase (DmpF) (25). DmpG and IPMS share the basic TIM-barrel catalytic domain and have nearly identical active site architecture, resulting in the DmpG structure as the best match to the catalytic domain using the secondary structure matching algorithm SSM (11). In addition, DmpG has a linker domain with an α -helical structure similar to that of IPMS. DmpG catalyzes the formation of acetaldehyde and pyruvate from 4-hydroxy-2-ketovalerate. The acetaldehyde is proposed to move through a 29 Å tunnel to the DmpF active site. Access to the tunnel is controlled by the presence of NAD^+ , a substrate for DmpF, at the DmpF active site coupled to the motion of residue Y291 on the linker domain of DmpG. When the structures of DmpG and *Mt*IPMS are superimposed, Y291 DmpG is found to be in a position similar to Y410 IPMS (Figure 5).

The results of kinetic and thermodynamic studies presented here are consistent with an allosteric network linking the catalytic and regulatory domains in *Mt*IPMS. The perturbation of the affinity of the enzyme for L-leucine caused by the E218A and Y410F substitutions, located 50 Å from the L-leucine binding site, implicates these residues as belonging to that network and broadly suggests that the interface

between the linker and catalytic domains contains important contacts in the network. More specifically, as the Y410F substitution has uncoupled the binding of L-leucine from changes at the active site, it is clear that the Y410F-containing loop is an integral part of the allosteric network in *Mt*IPMS. While the upstream connections of the allosteric network remain unidentified, Y410F IPMS now provides an excellent negative control for future structural studies aimed at more precisely defining the mechanism of interdomain communication in this biologically important enzyme.

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SUPPORTING INFORMATION AVAILABLE

Information regarding the results of the ultracentrifugation experiments, the multiple-sequence alignment, Eadie–Hofstee plots, and the 1H NMR spectrum of the reaction product. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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