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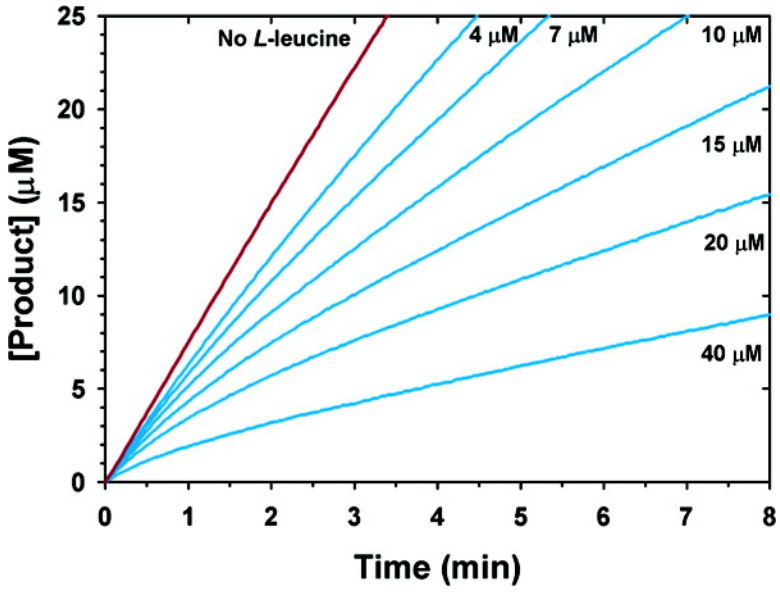

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Slow-onset Feedback Inhibition: Inhibition of *Mycobacterium tuberculosis* α-Isopropylmalate Synthase by L-Leucine

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α-Ketoisovalerate (KIV) is the biosynthetic precursor for L-leucine and L-valine, as well as pantothenate, the precursor of Coenzyme A (Supplementary Information). The first enzyme in the L-leucine biosynthetic pathway, the *leu*A-encoded α-isopropylmalate synthase has been shown to be feedback inhibited by the end-product of the pathway, L-leucine.[1−5] Recently, two crystal structures of α-isopropylmalate synthase from *Mycobacterium tuberculosis* (*Mt*IPMS) have been determined, one with bound Zn^{2+} and KIV and the other with bound L-leucine.[6] The active site is located in the N-terminal $(\alpha,\beta)_8$ barrel domain, while the L-leucine binding site is located in the C-terminal domain, more than 50 Å away from the active site. The only difference between these structures is a loop in the L-leucine binding site that gets ordered upon L-leucine binding. Thus, the mechanism of L-leucine inhibition cannot be deduced from these structures because the active site remains unaltered when L-leucine binds.

L-Leucine exhibits linear, noncompetitive inhibition versus KIV under initial velocity conditions, with K_{is} and K_{ii} values of 8 ± 1 μM and 22 ± 2 μM, respectively (Supporting Information). The small differences in the K_{is} and K_{ii} values indicate that the affinities of the free enzyme and substrate-bound enzyme for L-leucine are very similar. We found no kinetic evidence for cooperativity, suggesting that the two active sites as well as the two L-leucine binding sites in the native homodimer behave identically, which is also supported by the crystallographic data.[6] During the analysis of these data, it became apparent that the time courses of product formation were nonlinear and were exhibiting a time-dependent inhibition suggestive of slow-onset inhibition[7−10] (Figure 1). This type of kinetic behavior is usually due to a two-step binding process characterized by the rapid formation of an initial inhibitory complex, followed by a slower isomerization to a more tightly bound complex. The resulting time-courses display a burst of product formation followed by a linear steady-state phase (Figure 1). This pattern is a hallmark of slow-onset inhibition and has been observed in a number of enzymatic systems where inhibitors bind competitively versus the substrate.[7]

The time courses in Figure 1 were fitted to eq 1, where $[P]_t$ is the concentration of product formed (Coenzyme A) at time, t, v_i is the initial reaction rate, v_f the final reaction rate, and k_b the burst rate.[7,11]

$$[P]_t = v_f t + \frac{v_i - v_f}{k_b}(1 - e^{-k_b t}) \qquad (1)$$

Plotting the fitted values of k_b versus the [L-leucine] yields a hyperbolic dependence of k_b on [L-leucine] with a nonzero y-intercept value (Figure 2). The hyperbolic dependence of k_b on the [L-leucine] indicates that L-leucine inhibition can be described

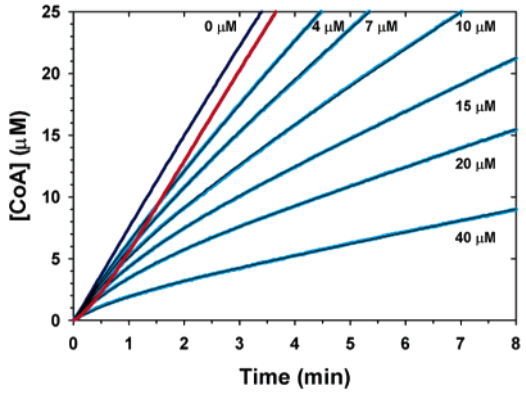

Figure 1. Kinetics of slow-onset inhibition of *Mt*IPMS by L-leucine. Reactions were initiated by the addition of enzyme. Solid lines represent data obtained in the absence (blue) and in the presence of L-leucine (cyan). Black lines represent the fit to eq 1. Concentrations of L-leucine are indicated. Experimental methods are described in Supporting Information. The red line represents an experiment where the reaction was initiated by the addition of *Mt*IPMS preincubated with L-leucine.

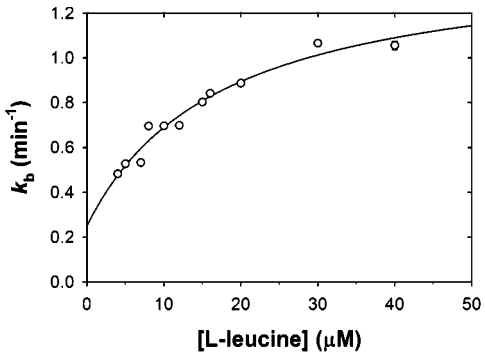

Figure 2. Dependence of the burst rate (k_b) on [L-leucine]. The points represent data, and the solid line represents the fit to eq 2.

Scheme 1

$$E + Leu \underset{}{\overset{k_1}{\rightleftharpoons}} E{\cdot}Leu \overset{k_2}{\rightleftharpoons} E^*{\cdot}Leu$$

by the rapid formation of an initial E·Leu complex followed by an isomerization event that generates a more tightly bound E*·Leu complex (Scheme 1). As all experiments were performed at saturating concentrations of both substrates (see below), E in Scheme 1 represents the ternary E·α-KIV·AcCoA complex, and not free enzyme. This two-step binding model can be described by eq 2, where k_{-2} is the reverse rate for the isomerization, k_2 is the forward rate for the isomerization, K_i is the inhibition constant for L-leucine and [L] the concentration of L-leucine.

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$$k_b = k_{-2} + \frac{(k_2 + k_{-2})[L]}{K_i + [L]} \quad (2)$$

$$K_i = \frac{k_{-1}}{k_1} \quad (3)$$

$$K_i^* = K_i \frac{k_{-2}}{k_2 + k_{-2}} \quad (4)$$

The data in Figure 2 were fitted to eq 2 giving values for k_2 of 0.95 ± 0.12 min^{-1}, k_{-2} of 0.25 ± 0.1 min^{-1}, and K_i of 17 ± 8 μM, which is in excellent agreement with the K_{ii} value (22 μM: L-leucine binding to E·α-KIV·AcCoA rather than E), determined independently from steady-state initial velocity analysis (above). Finally, the value of 3.6 ± 2.2 μM for K_i^* was calculated from eq 4.[12]

Slow-onset inhibition may also be studied by preincubation of the enzyme and the competitive inhibitor followed by addition of the substrate at the start of the reaction, which generates a lag phase followed by a steady-state rate.[7] No lag is seen by initiating the reaction with substrates since L-leucine remains bound at the allosteric site (data not shown). To document the reversibility of the inhibition by L-leucine, we preincubated 5 μM enzyme with 25 μM L-leucine on ice for 15 min to generate the E*·Leu complex. Subsequently, this complex was diluted 250-fold into the reaction mixture (Figure 1, red line). The observation of a lag upon dilution of the inhibited complex and the similarity of the steady-state rate of the reaction performed without L-leucine (blue solid line) indicates that the inhibition by L-leucine is reversible. The presence of substrates in preincubation mixtures had no effect and is thus not required to form the tightly inhibited complex (data not shown).

The structural basis for the inhibition by L-leucine and the slow-onset nature of the inhibition are unclear at this juncture. However, it is clear from the structure that the inhibitory signal must be transmitted through the domains that link the regulatory and catalytic domains. One possible explanation for the slow-onset nature of the inhibition involves the ordering of the loop in the L-leucine binding site,[6] leading to tighter binding of L-leucine. This model would account for both the rapid initial binding as well as the slower isomerization to the more tightly bound form.

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Supporting Information Available: L-Leucine biosynthetic pathway, experimental procedures, and inhibition patterns are described. This material is available free of charge via the Internet at http://pubs.acs.org.

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