Molecular basis of the inhibitor selectivity and insights into the feedback inhibition mechanism of citramalate synthase from Leptospira interrogans

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LiCMS (Leptospira interrogans citramalate synthase) catalyses the first reaction of the isoleucine biosynthesis pathway in L. interrogans, the pathogen of leptospirosis. The catalytic reaction is regulated through feedback inhibition by its end product isoleucine. To understand the molecular basis of the high selectivity of the inhibitor and the mechanism of feedback inhibition, we determined the crystal structure of LiCMS (C-terminal regulatory domain of LiCMS) in complex with isoleucine, and performed a biochemical study of the inhibition of LiCMS using mutagenesis and kinetic methods. LiCMS forms a dimer of dimers in both the crystal structure and solution and the dimeric LiCMS is the basic functional unit. LiCMS consists of six β-strands forming two anti-parallel β-sheets and two α-helices and assumes a βαβ three-layer sandwich structure. The inhibitor isoleucine is bound in a pocket at the dimer interface and has both hydrophobic and hydrogen-bonding interactions with several conserved residues of both subunits. The high selectivity of LiCMS for isoleucine over leucine is primarily dictated by the residues, Tyr430, Leu451, Tyr454, Ile458 and Val468, that form a hydrophobic pocket to accommodate the side chain of the inhibitor. The binding of isoleucine has inhibitory effects on the binding of both the substrate, pyruvate, and coenzyme, acetyl-CoA, in a typical pattern of K-type inhibition. The structural and biochemical data from the present study together suggest that the binding of isoleucine affects the binding of the substrate and coenzyme at the active site, possibly via conformational change of the dimer interface of the regulatory domain, leading to inhibition of the catalytic reaction.

Key words: allosteric regulation, crystal structure, feedback inhibition, mechanism, selectivity.

INTRODUCTION

Leptospirosis is a zoonotic infectious disease which occurs commonly in the tropics with favourable conditions for transmission, but can also occur in urban areas of industrialized and developing countries (reviewed in [1,2]). A major and often lethal manifestation of leptospirosis is endemic and epidemic severe pulmonary haemorrhage. Leptospirosis is caused by Leptospira interrogans, which belongs to spirochetes, a group of bacteria that diverged in the early stage of bacterial evolution [3]. The metabolism of spirochetes differs substantially from that of few other microorganisms such as Mycobacterium tuberculosis [8].

During the metabolism of microorganisms, many enzymes regulate their enzymatic activities through binding of effectors at distinct allosteric sites away from the active sites. Usually, the end-products of the metabolic pathways function as feedback inhibitors, and thus the regulation process is called feedback inhibition. The mechanism of feedback inhibition has been studied for several important enzymes at both structural and biochemical levels. For examples, aspartate kinases catalyse the biosynthesis of threonine, lysine, and methionine from aspartate, which can be inhibited by its end-products (threonine, lysine and methionine respectively) [9–12]. The binding of lysine to the regulatory domain induces conformational change of the catalytic domain, preventing the binding of the substrate ATP, at the active site and thus inhibiting the enzymatic activity. ATP-PRTase (ATP-phosphoribosyltransferase) catalyses the condensation of ATP with 5′-phosphoribosyl 1′-pyrophosphate, the first reaction of the histidine biosynthetic pathway, and the reaction is regulated by its end-product histidine [13,14]. The binding of His to ATP-PRTase causes a major conformational change of the dimer interface, leading to formation of a stable, inactive hexamer. In most microorganisms, threonine dehydratase catalyses the first reaction of the biosynthesis of isoleucine from threonine and the reaction is subjected to feedback inhibition by its end-product, isoleucine [15–17]. The binding of isoleucine to the regulatory domain induces conformational changes of the dimer interface and thus
affects the transition of the enzyme from the high active state to the low active state.

The biosynthesis of isoleucine in L. interrogans is very similar to the biosynthesis of leucine in most microorganisms. IPMS (α-isopropylmalate synthase) catalyzes the first reaction of the leucine biosynthetic pathway, the synthesis of α-isopropylmalate from α-ketoisovalerate and acetyl-CoA, and the reaction is inhibited by the end-product leucine [18–20]. The structure of MtIPMS (IPMS from Mycobacterium tuberculosis) has shown that the enzyme consists of a catalytic domain at the N-terminus and a regulatory domain at the C-terminus linked together by two small domains and a disordered loop [18]. The regulatory domain of MtIPMS assumes a βαβ three-layer sandwich structure which is different from the regulatory domains of other allosteric enzymes. Although the structural and biochemical data of MtIPMS have provided some insights into the mechanism of feedback inhibition, the exact details of the mechanism are unclear [19,20].

Similar to MtIPMS, LiCMS also consists of an N-terminal catalytic domain (residues 1–330, LiCMSN) and a C-terminal regulatory domain (residues 390–516, LiCMSC) linked together by a flexible region of approx. 60 residues. Previously we have described the crystal structures of the catalytic domain of LiCMS in complex with substrate and coenzyme, which together with the biochemical data revealed the mechanism of the catalytic reaction of LiCMS [21]. We report in the present study the crystal structure of the regulatory domain of LiCMS in complex with its end-product isoleucine and the biochemical studies of the inhibition of LiCMS by isoleucine. The structural and biochemical data together reveal the molecular basis of the high selectivity for isoleucine over leucine by LiCMS and provide insights into the mechanism of feedback inhibition of LiCMS.

**MATERIALS AND METHODS**

**Expression and purification of the full-length LiCMS**
Expression and purification of the full-length LiCMS were carried out as described [21]. Constructs of the mutant LiCMS containing point mutation(s) were generated using the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene) and were verified by DNA sequencing. Expression and purification of the wild type LiCMS were the same as the wild-type enzyme.

Gel-filtration analysis of the purified protein was performed to measure the oligomeric state of LiCMS in solution. The high molecular weight (mass) Calibration Kit (GE-Healthcare) was used to calibrate the molecular mass of LiCMS. All analyses were carried out on an FPLC Purifier (Amersham Bioscience). Protein samples were loaded into a 200 ml sample loop and injected into a Superdex G200 preparation column. The apparent molecular mass of the protein sample was calculated according to the protocol provided in the kit.

**Enzymatic activity assay of LiCMS**
The enzymatic activity assay of both wild-type and mutant LiCMS was carried out as described previously [21]. In the measurements of the inhibition curve and IC_{50} value (the concentration of the inhibitor inhibiting 50 % of the enzymatic activity) of LiCMS by isoleucine and its analogue leucine, the reaction mixture consisted of 0.6 mM Pyr, 4 mM acetyl-CoA, 0.8 mM MnCl_{2}, 50 mM KCl, 15 mM LiCMS and a varied concentration of isoleucine (approx. 0–10 mM) or leucine (approx. 0–20 mM) in a total volume of 50 µl using 0.1 M Hepes (pH 7.7). The inhibition curve was obtained by fitting the kinetic data to the equation of one-phase exponential association or the equation of one-phase exponential decay. The IC_{50} value was derived from the one-phase exponential decay equation. The apparent inhibition constant K_i was calculated from the Cheng–Prusoff equation for competitive inhibition on the binding of acetyl-CoA [22]. The inhibition type of wild-type LiCMS by isoleucine was determined based on analysis of the changes of substrate-binding affinity and turnover number at different concentrations of isoleucine. In this measurement, the reaction mixture consisted of 0.8 mM MnCl_{2}, 50 mM KCl, 15 mM LiCMS, a fixed concentration of Pyr (2 mM) and a varied concentration of acetyl-CoA (0.2–4 mM) or a varied concentration of Pyr (0.04–0.8 mM) and a fixed concentration of acetyl-CoA (4 mM), at different concentrations of isoleucine (0–60 µM) in a total volume of 50 µl using 0.1 M Hepes (pH 7.7). The kinetic data was fitted to the Scatchard plot for apparent K_i and approximate V_{max}, and the k_{cat} value was calculated from V_{max}. All kinetic data were analysed using the program Prism 4.0 (GraphPad Software).

**Crystallization and diffraction data collection**
The full-length LiCMS protein was used in all crystallization experiments, which were carried out using the hanging-drop vapour diffusion method. In the crystallization of both wild-type and SeMet-substituted LiCMS at room temperature (approx. 20°C), two types of crystals were grown from the same crystallization drop consisting of equal volumes (2 µl) of the protein solution (approx. 20 mg/ml of LiCMS in 20 mM Tris/HCl, pH 8.4, and 50 mM NaCl) and the reservoir solution (0.1 M Hepes, pH 7.5, and 2.0 M sodium malonate) [21]. SDS/PAGE analyses indicate that the full-length LiCMS was hydrolysed into two fragments during the crystallization: the larger fragment corresponds to LiCMSN and the smaller fragment to LiCMSC. Crystals of a tetragonal bipyramidal shape contain the larger fragment LiCMSN and crystals of a hexagonal plate shape contain the smaller fragment LiCMSC. The crystals of LiCMSC growing at this condition belong to space group C222 (type I) with the unit cell parameters a = 62.6 Å (1 A = 0.1 nm), b = 98.4 Å and c = 40.1 Å. There is one LiCMSC molecule in the asymmetric unit, corresponding to a Matthews coefficient (V_{M}) of 2.3 Å³/Da and a solvent content of 45.5 %. Later on, two new types of crystals of LiCMS (types II and III) were grown from the crystallization solution containing equal volumes of the protein solution (approx. 20 mg/ml of LiCMS in 20 mM Tris/HCl, pH 8.4, and 50 mM NaCl) and the reservoir solution (0.1 M Tris/HCl, pH 8.0, and 1.6 M (NH₄)₂SO₄). But no crystal of LiCMSCN was grown at this new condition. The type II crystals of LiCMSC were grown at room temperature and belong to space group C2 with the unit cell parameters a = 61.3 Å, b = 97.9 Å, c = 40.0 Å and β = 91.4°. There are two LiCMSC molecules in the asymmetric unit, corresponding to a Matthews coefficient of 2.2 Å³/Da and a solvent content of 43.9 %. The type III crystals of LiCMSC were grown at 4°C and belong to space group C222 with the unit cell parameters a = 108.2 Å, b = 118.6 Å and c = 63.6 Å. There are two LiCMSC molecules in the asymmetric unit, corresponding to a Matthews coefficient of 3.7 Å³/Da and a solvent content of 67.0 %. These three types of crystals are closely related to each other with slight differences in crystal packing.

The crystals of LiCMSC used for diffraction data collection were first cryo-protected using paratone oil (Hampton) and then dipped into liquid nitrogen. The MAD (multiple-wavelength anomalous dispersion) diffraction data were collected to 2.5 Å resolution from a flash-cooled SeMet-substituted LiCMSC crystal (type I) at 100 K at Photon Factory beamline BL6A, and processed and scaled together using the HKL2000 suite [23]. A native
Table 1  Summary of diffraction data and structure refinement statistics

<table>
<thead>
<tr>
<th>Crystal type</th>
<th>Type I</th>
<th>Type II</th>
<th>Type III</th>
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<tr>
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<td>Peak</td>
<td>Edge</td>
<td>Remote</td>
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</tr>
<tr>
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<td>50.0–2.50 (2.59–2.50)</td>
<td>50.0–2.50 (2.59–2.50)</td>
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<tr>
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<td>C222</td>
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<tr>
<td>Cell parameters</td>
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<td>c (Å)</td>
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<tr>
<td>β (°)</td>
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<td>91.4</td>
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<td>13.7 (12.8)</td>
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<tr>
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<td>99.5 (96.1)</td>
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<td>7.2 (39.6)</td>
<td>8.1 (46.2)</td>
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Statistics of refinement and model

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<td>Most favoured regions</td>
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The structure of LiCMS was solved with the MAD method implemented in the program SOLVE [25]. SOLVE with the default parameters identified three Se atoms, indicating that there is one LiCMS molecule in the asymmetric unit because LiCMS contains three methionine residues. The MAD phases were improved by statistical density modification using the program RESOLVE [26], increasing the overall figure-of-merit from 0.55 to 0.67 at 2.5 Å resolution. RESOLVE automatically built a partial polyalanine model (about 40%) of the protein, which is relatively low probably because LiCMS is composed largely of β-strands. The MAD-phased electron density map was of high quality to allow manual building of a full structure model. This structure model was used as a template to solve the structure of LiCMS in type II and III crystals using the molecular replacement method implemented in the program CNS [27]. Structure refinement of the protein model was performed using CNS and manual model building was performed using the program Coot [28]. In both structures, there was strong electron density at two different locations of the dimer interface which could be fitted with an isoleucine and a divalent metal ion respectively. Since neither isoleucine nor divalent metal ion was added in the crystallization solution, the bound isoleucine and metal ion must be co-purified with the enzyme from the expression system. In our previous work, we carried out ICP-MS (inductively coupled plasma MS) analysis of the purified full-length LiCMS, and the results showed that the purified protein contains both Zn2+ and Ni2+, and the bound Zn2+ was co-purified with the enzyme from the expression system and the concomitant Ni2+ was stripped from the Ni-NTA (Ni2+-nitrilotriacetate) column during purification [21]. Similar to the structure determination of LiCMSN, we performed an X-ray fluorescence scan of the LiCMS crystal and collected anomalous diffraction data at the Zn and Ni wavelengths using synchrotron radiation source. The results show that there is strong anomalous density (above 10σ contour level) for a Zn2+
ion at the dimer–dimer interface (Supplementary Figure S1 at http://www.BiochemJ.org/bj/421/bj4210133add.htm), but no anomalous density for a Ni$^{2+}$ ion. Thus, we defined the bound metal ion as Zn$^{2+}$. The statistics of the structure refinement and the quality of structure models are summarized in Table 1.

RESULTS AND DISCUSSION

Overall structure of LiCMSC

The crystal structure of LiCMSC was solved using the MAD method. The structure of LiCMSC was refined against a 2.0 Å resolution dataset of the type II crystal (space group C2) yielding an $R$ factor of 22.8% and a free $R$ factor of 26.2%. and a 2.7 Å resolution dataset of the type III crystal (space group C222), yielding an $R$ factor of 23.9% and a free $R$ factor of 27.4% respectively (Table 1). The type II crystal structure contains two LiCMSC molecules, two isoleucine molecules, two Zn$^{2+}$ ions (which are located on the two-fold symmetry axis with an occupancy of 0.5) and eight SO$_4^{2-}$ ions in the asymmetric unit. The overall structures of the two LiCMSC monomers are very similar, with a RMSD (root mean square deviation) of 0.48 Å. The structure model of LiCMSC contains residues 390–512, with residues 478–479 and 513–516 disordered (there are some variations of the disordered regions in the two subunits). Similar to the regulatory domain of MiIPMS, LiCMSC consists of six β-strands forming two anti-parallel β-sheets (β1–β3 and β4–β6) and two α-helices (α1 and α2) and assumes a three-layer sandwich structure (Figure 1A). The two β-sheets are nearly orthogonal to each other and form the bottom and top layers, and the two α-helices are sandwiched in the middle. The two LiCMSC molecules (A and B) in the asymmetric unit form a tight dimer in a head-to-head manner, in which the two β-sheets of one monomer are anti-parallel to those of the other monomer to form two six-stranded β-sheets (β1–β3 and β1′–β3′, and β4–β6 and β4′–β6′) (for simplicity, structural elements and residues from the adjacent monomer are indicated with an apostrophe hereafter), and the two α-helices of one monomer extend to those of the other monomer to form two pseudo long α-helices arranged as an ‘X’ shape. The dimer interface contains extensive hydrophilic and hydrophobic interactions and the dimerization buries 1583 Å$^2$ or 22.8% of the solvent accessible surface area of each monomer. The two isoleucine molecules are bound at the dimer interface and interact with residues from both subunits (see discussion later). In addition, two head-to-head dimers related by the 2-fold symmetry pack against each other in a side-by-side manner to form a tetramer (Figure 1B). Thus in the discussion below, the type II structure will be used for further structural analysis because of the higher resolution.

LiCMS functions as a dimer

Previously, our structural and biochemical data have shown that the catalytic active site of LiCMS is composed of structural elements from both subunits of a dimer and LiCMS appears to function as a dimer [21]. It is very intriguing to observe that in the structures of LiCMSC, two head-to-head dimers are related by a 2-fold symmetry to form a tetramer and there are two Zn$^{2+}$ ions bound at the side-by-side dimer interface of the tetramer, each of which is co-ordinated by four histidine residues from two adjacent subunits and two water molecules (Figure 1C). Thus, we raised the questions whether the metal ions bound at the side-by-side interface have any functional role, whether the binding of isoleucine plays any role in the formation of the tetramer, and whether LiCMS functions biologically as a dimer or tetramer. To address these questions, we performed gel filtration analyses of the wild-type and mutant LiCMS and determined their enzymatic activities and inhibitory effects by isoleucine. Gel filtration analyses show that the purified wild-type LiCMS exists dominantly as a tetramer in solution presumably due to the presence of Zn$^{2+}$ co-purified with the enzyme from the expression system (Figure 1D). To remove the bound metal ions, we added excess EDTA into the purified protein solution and then dialysed it against a buffer (20 mM Tris/HCl, pH 8.0) containing 10 mM EDTA. The resultant LiCMS is determined to exist mainly as a dimer (Figure 1D). However, removal of EDTA from the protein solution by dialysis against the buffer led to the enzyme existing as a mixture of both dimer and tetramer, presumably due to the presence of a trace of divalent metal ion(s) in the dialysis buffer (results not shown). To disrupt the binding of the two Zn$^{2+}$ ions at the side-by-side dimer interface of the tetramer, we mutated His$^{400}$ and His$^{408}$, that are involved in the co-ordination of Zn$^{2+}$ on alanine. The H400A/H408A mutant LiCMS is shown to exist purely as a dimer in solution (Figure 1D). We tested further the enzymatic activity of the H400A/H408A mutant LiCMS and its inhibition by isoleucine. The kinetic data show that the mutant LiCMS is inhibited by isoleucine with comparable IC$_50$ and K$_i$ values as the wild-type enzyme (Table 2A), and the double mutations have very little effects on the binding (K$_i$ values) of acetyl-CoA and the k$_{cat}$ (Table 2B). In addition, binding of isoleucine with the mutant LiCMS does not affect the dimeric state of the enzyme (results not shown). These results indicate that the dimeric LiCMS is the basic functional unit and the binding of isoleucine does not
Structure of the regulatory domain of LiCMS

Figure 1  For legend see next page
alter the oligomeric state of the enzyme. The binding of metal ions at the side-by-side dimer interface leads to the formation of the tetramer but has no effect on the enzymatic activity and the inhibition by isoleucine.

Structure of the inhibitor-binding site
In the crystal structure of LiCMSC, there are two isoleucine molecules bound at the head-to-head dimer interface, even though no isoleucine was added in the crystallization solution, suggesting that isoleucine has a tight binding to LiCMSC. The inhibitor-binding site is formed by several conserved residues from the β6-α2 turn and the β4-β5 loop of one monomer and the β3-α1 turn and β4 of the other monomer (Figure 1A). The inhibitor isoleucine has both hydrogen-bonding and hydrophobic interactions with the surrounding residues (Figures 2A and 2B). Specifically, one carboxyl oxygen of isoleucine forms two hydrogen bonds with the main-chain amide of Glu208 (2.7 Å) and a
Structure of the regulatory domain of LiCMS

Figure 2 For legend see next page
When the residues of LiCMS were changed to the equivalent residues of MtIPMS, there would be no steric conflict between the docked leucine and the surrounding residues. The van der Waals interaction of the docked leucine (mainly the Cδ2 atom) would have steric hindrance with the surrounding residues (particularly Ile458 and Val468) (Figure 2D, left panel). When the residues of LiCMS are changed to the corresponding residues of MtIPMS, there is no steric conflict between the docked leucine and the surrounding residues (Figure 2D, right panel). These results suggest that the high selectivity of LiCMS for isoleucine over leucine appears to rely on these hydrophobic residues. To verify this notion, we generated a number of LiCMS mutants in which the residues forming the hydrophobic pocket are substituted with their counterparts in MtIPMS (except Y454A) and determined the enzymatic activities of these mutants and their inhibition by isoleucine. The kinetic data show that these mutations (particularly Y454A and I458A) had minor effects on the $K_a$ for acetyl-CoA but caused moderate decreases of the $k_{cat}$ in the absence of isoleucine (Table 2B), suggesting that changes at the inhibitor-binding site can affect the catalytic reaction of LiCMS. Furthermore, these mutations had varied effects on the inhibition of LiCMS by isoleucine (Figure 3D and Table 2A). Except the V468A mutant, which has a slightly increased ($<2$-fold) inhibition compared with that of the wild-type enzyme, the inhibition of all other mutants (Y430L, L451V, Y454A and I458A) by isoleucine is severely decreased with markedly increased $k_{cat}$ and $K_i$ values, and their enzymatic activities are less sensitive to low concentration of isoleucine, indicating that these mutations significantly weaken the binding of isoleucine to LiCMS. On the other hand, since mutations of these residues to their counterparts in MtIPMS would create a hydrophobic pocket suitable for binding leucine, it is expected that some of these LiCMS mutants would have the ability to bind both isoleucine and leucine and thus can be inhibited by both inhibitors. To test this hypothesis, we also determined the inhibition of these mutants by leucine. Indeed, the V468A mutant can be substantially inhibited by leucine and the Y454A mutant to a less significant extent (Figure 3E and Table 2A).

These biochemical data can be explained by the crystal structure of LiCMS. In the structure of LiCMS in complex with isoleucine, Val468 is located about 4.0Å from the side-chain Cγ2 atom of isoleucine. Mutation of Val468 to alanine would have a very subtle effect on the binding of isoleucine and thus the inhibition of this mutant by isoleucine is slightly increased. On the other hand, since the side chain of Val468 has steric conflict with the side-chain Cγ2 atom of the docked leucine, its mutation to alanine would eliminate the steric hindrance and allow the binding of leucine. Thus, leucine becomes an effective inhibitor of the V468A mutant. Residue Tyr454 has contacts with the Cγ2 and Cδ1 atoms of isoleucine and its mutation to alanine would create a larger pocket that can accommodate both isoleucine and leucine with weak binding affinities. Therefore, the Y454A mutant could still be inhibited by isoleucine at a substantially impaired level, as well as be weakly inhibited by leucine. Residue Tyr454 has hydrophobic contacts with the side-chain Cγ2 and Cγ2 atoms of isoleucine and its mutation to leucine leads to loss of the contacts with the inhibitor and decrease of the inhibitor binding, explaining

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**Figure 2** Structure of the inhibitor-binding site

(A) Structure of the inhibitor-binding site showing the interactions between the inhibitor isoleucine (magenta) and the surrounding residues (gray). The hydrogen-bonding interactions are indicated with dashed lines. (B) The hydrophobic pocket for the side chain of the inhibitor is composed of several hydrophobic residues (yellow) and is covered by the β4–β5 loop (cyan). (C) Structure-based sequence alignment of the regulatory domains of LiCMS and MtIPMS. The secondary structure elements of LiCMS and MtIPMS are shown on top of and below the alignment respectively. Residues that have hydrogen-bonding interactions with isoleucine are marked with red triangles, and residues that have hydrophobic interactions with the side chain of isoleucine are marked with red stars. Strictly conserved residues are highlighted in shaded red boxes and conserved residues in open red boxes. (D) Selectivity of LiCMS for isoleucine over leucine. Left: when leucine (magenta) is docked into the inhibitor-binding site of LiCMS, the side chain of leucine (mainly the Cα2 atom) would have steric conflicts with the surrounding residues (particularly Ile458 and Val468) (cyan). Right: when the residues of LiCMS were changed to the equivalent residues of MtIPMS, there would be no steric conflict between the docked leucine and the surrounding residues. The van der Waals spheres of atoms are shown with dotted meshes.

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Figure 3  For legend see next page
why the inhibition of the Y430L mutant by isoleucine is severely impaired. Leu431 has contacts with the Cα1 atom of isoleucine and its mutation to valine has a moderate effect on the binding of isoleucine and thus a less severe impact on the inhibition of LiCMS. Residue Ile439 is located on the β4–β5 loop, which covers the top of the hydrophobic pocket for the side chain of isoleucine and has contacts with the Cy1 and Cα1 atoms of isoleucine. Mutation I458A completely disrupts the binding of isoleucine and abolishes the inhibition of LiCMS by isoleucine. It is possible that the impairment of the inhibitor binding by this mutation might be through conformational change of the β4–β5 loop.

Furthermore, we generated a number of LiCMS mutants containing point mutations of these residues that have hydrogen-bonding interactions with the amino and carboxyl groups of isoleucine (including Asp931, Thr932, Pro933 and Gln935), with alanine residues, except Ala886 (which is already an Ala) and Tyr880 (which has been discussed above), and determined the enzymatic activities of these mutants and their inhibition by isoleucine. All of these mutations caused a moderate decrease in IC50 and K\text{m} values because the change of electrostatic property of the residue would affect the binding of isoleucine. The T464A mutation has a minor effect on the IC50 and K\text{m} values because its side chain has no interaction with isoleucine and the change has no significant effect on the hydrogen-bonding interaction between its main-chain carbonyl group and the main-group carboxy group of isoleucine. The inhibition of the P493A mutant by isoleucine is greatly decreased. Pro993 is located on the β6–α2 turn and it is possible that substitution of Pro993 with alanine could increase the flexibility of the turn and thus affect the binding of isoleucine. Gln995 forms two hydrogen bonds with one carboxyl oxygen of isoleucine: one from the main-chain amide group and the other from the side-chain amino group via a water molecule. The Q495A mutant exhibits significantly increased IC50 and K\text{m} values, probably because the mutation would abolish the hydrogen-bonding interaction mediated by the water molecule. These results clearly show that these mutations can significantly weaken the binding of isoleucine and thus severely impair the inhibition of LiCMS by isoleucine. On the other hand, none of these mutants is sensitive to leucine, indicating that these residues make no notable contribution to the selectivity of LiCMS for isoleucine over leucine. This is expected because these mutations would retain most of the hydrogen-bonding interactions between their main chains and the inhibitor and thus would play no major role in determining the side chain of the inhibitor. Taking the structural and biochemical data together, we conclude that the high selectivity of LiCMS for isoleucine over leucine is dictated by the residues forming the hydrophobic pocket to accommodate the side chain of the inhibitor.

**Insights into the mechanism of feedback inhibition of LiCMS by isoleucine**

In the metabolic pathways, many enzymes are subjected to feedback regulation by their end-products bound at allosteric sites distinct from the active sites, and the allosteric sites are usually located at the dimer interface of their regulatory domains. The binding of an inhibitor can cause conformational change of the dimer interface, which could be relayed to the active site through flexible inter-domain linkers [10–12,16,29,30] or could lead to a change in the oligomeric states of the enzymes [14]. The enzymatic activity of LiCMS is regulated by the end-product isoleucine via a feedback inhibition. Analyses of the available structural and biochemical data provide some insights into the mechanism of feedback inhibition of LiCMS by isoleucine. Our previous structural data have shown that the catalytic domain LiCMSN exists as a dimer in the crystal structure and the active site consists of structural elements of both subunits [21]. The structural data presented here show that the regulatory domain of LiCMSN forms a dimer of two head-to-head dimers in the crystal structure and the inhibitor-binding site is located in the head-to-head dimer interface and composed of structural elements of both subunits as well (Figure 2). The biochemical data show that LiCMS functions as a dimer and the binding of isoleucine does not disrupt the dimerization or cause higher oligomerization of LiCMS. Thus, one possibility for the inhibition of LiCMS by isoleucine is that, similar to most other allosteric enzymes, the binding of isoleucine to the regulatory domain of LiCMS might cause conformational change of the dimer interface of the regulatory domain, which further induces conformational changes of the catalytic domain and the active site and thus affects the binding of acetyl-CoA and/or Pyr, leading to inhibition of the catalytic reaction. This hypothesis is supported by our biochemical data that the binding of isoleucine can affect the binding affinities of both acetyl-CoA and Pyr in a concentration-dependent manner, and mutations of the residues at the inhibitor-binding site can cause substantial decrease of the binding of isoleucine and moderate decrease of the k\text{m}. (Figure 3 and Table 2). Nevertheless, because LiCMS was co-purified with isoleucine bound, we could not obtain the structure of LiCMS or LiCMSN in the absence of isoleucine and thus do not know exactly the conformational change of the dimer interface of the regulatory domain caused by the binding of isoleucine. Structural determination of the apo form of LiCMSN and in particular the full-length LiCMS in both the presence and absence of the inhibitor will eventually resolve the issue.

**Figure 3** Inhibition of the enzymatic activity of LiCMS by the feedback inhibitor isoleucine

(A) Inhibition curve of the enzymatic activity of LiCMS with increased concentration of isoleucine. (B) Scatchard plot of acetyl-CoA binding to LiCMS at different concentrations of isoleucine. (C) Scatchard plot of Pyr binding to LiCMS at different concentrations of isoleucine. (D) Effects of mutations of the residues that have hydrophobic interactions with the side chain of the inhibitor on the inhibition of LiCMS by isoleucine. (E) Effects of mutations of the residues that have hydrophobic interactions with the side chain of the inhibitor on the inhibition of LiCMS by isoleucine. (F) Effects of mutations of the residues that have hydrogen-bonding interactions with the inhibitor on the inhibition of LiCMS by isoleucine. Most of these mutations cause decreased inhibition of LiCMS by isoleucine, and mutations Y468A and Y454A can confer inhibition by leucine on the mutant enzyme.

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AUTHOR CONTRIBUTIONS
Peng Zhang and Manwu Zha performed the structural studies; Jun Ma performed the biochemical studies; Zilong Zhang and Hai Xu participated in the biochemical studies; Jiaping Ding and Guoping Zhao designed the research; Jiaping Ding, Peng Zhang and Jun Ma wrote the paper.

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SUPPLEMENTARY ONLINE DATA

Molecular basis of the inhibitor selectivity and insights into the feedback inhibition mechanism of citramalate synthase from *Leptospira interrogans*

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Figure S1  Anomalous electron density of the bound Zn$^{2+}$ ion

There are strong anomalous density peaks (above 10σ contour level) for the bound Zn$^{2+}$ ions at the dimer–dimer interface of the LiCMSC tetramer.

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The atomic and structural coordinates of the regulatory domain of *Leptospira interrogans* citramalate synthase in complex with isoleucine in the crystal types II (C2) and III (C222) have been deposited in the Protein Data Bank under accession codes 3F6G and 3F6H respectively.

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