Subdomain II of α-Isopropylmalate Synthase Is Essential for Activity

INFERRING A MECHANISM OF FEEDBACK INHIBITION*

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4 The abbreviations used are: IPMS, α-isopropylmalate synthase; Lb, L. biflexa; α-Kiv, α-ketoisovalerate; α-IPM, α-isopropylmalate; Mt, M. tuberculosis; Li, L. interrogans; HS-CoA, reduced CoA; CMS, citramalate synthase; Sc, S. cerevisiae; Nm, N. meningitidis; SpHCS, homocitrullate synthase from S. pombe; TIM, triosephosphateisomerase.

Background: Isopropylmalate synthases (IPMSs) with and without a regulatory domain were found.

Results: IPMS subdomain II is essential for activities and likely involved in acetyl-CoA binding-mediated conformation transition.

Conclusion: The N-terminal domain and the two subdomains comprise the complete and independently functional catalytic module of IPMS.

Significance: The IPMS catalytic module was defined and characterized, which inferred a probable feedback inhibition mechanism.

The biosynthesis of branched chain amino acids usually starts from an aldol condensation reaction catalyzed by an α-ketosynthase, which is also usually an allosteric enzyme subjected to feedback inhibition by its corresponding end product (1–4). As a typical representative, α-isopropylmalate synthase (EC 2.3.3.13; IPMS)4 catalyzes the committed step of leucine biosynthesis, i.e. converting acetyl-CoA and α-ketoisovalerate (α-Kiv) to α-isopropylmalate (α-IPM), followed by a series of reactions catalyzed by α-isopropylmalate isomerase, α-isopropylmalate dehydrogenase, and aminotransferase, leading to the synthesis of the end product L-leucine (5–8).
The crystal structure of IPMS from Mycobacterium tuberculosis (MtIPMS) (9) revealed an asymmetrical homodimer architecture. Each monomer is composed of an N-terminal domain and a C-terminal regulatory domain connected by two subdomains, designated as I and II. The N-terminal domain assumes a (β/α)8 TIM barrel with one Asp and two His residues located at the active site to bind the substrate α-Kiv and a Zn2+ ion. Based on the structural results, a possible four-step catalytic mechanism was proposed with enol formation and stabilization mediated by interaction with the positively charged side chain of Arg-80 as one of the critical steps (9, 10). Later, the crystal structure of the N-terminal domain of (R)-citramalate synthase (EC 2.3.1.182; CMS) from Leptospira interrogans (LiCMSN), an analogous allosteric enzyme for isoleucine bio-synthesis, in complex with acetyl-CoA identified the acetyl-CoA binding site, and the structural and biochemical data together revealed the molecular basis of the substrate specificity (11). In addition, the structure of the C-terminal regulatory domain of the same enzyme (LiCMSC) complexed with isoleucine clearly demonstrated the ligand-binding site and its selectivity for feedback inhibition (12).

One of the important questions raised during these studies is whether the regulatory domain is required for the catalytic activity (13, 14). Several recent studies seem to support the idea that the activity of IPMS is dependent on its C-terminal regulatory domain (13–16). However, as early as 2003, when the genome of L. interrogans serotype lai was initially sequenced, three copies of leuA-like genes were annotated (7). One of them, LA_0469 was proved to encode an active IPMS with a significantly shorter peptide than those encoded by the other two genes (LA_2350, i.e. cimA and LA_2202, i.e. leuA1); it apparently lacks the regulatory domain and thus was designated as leuA2. This finding implied that the catalytic activity of the IPMS-like enzymes might be independent of the regulatory domain and thus directly led us to characterize the enzymatic features of both the long and short forms of IPMSs from Leptospira biflexa. We determined the crystal structure of the short form IPMS. The following molecular and biochemical comparative analyses identified the minimal key structural elements responsible for the full activity of IPMS without the regulatory domain. Further structural comparison with MtIPMS revealed conformational difference between the long and the short forms of the enzymes, which may represent different stages of catalysis. Combining the previously published hydrogen-deuterium exchange data with our mutational analysis data, a potential mechanism involving acetyl-CoA binding mediated by the two subdomains and associated with acetyl-CoA binding is proposed for feedback inhibition.

**EXPERIMENTAL PROCEDURES**

Cloning, Expression, and Purification of LbIPMS—The LbIPMS gene (leuA) used for clone construction and protein expression was amplified by PCR from the genomic DNA of L. biflexa (serovar Patoc strain “Patoc 1 (Paris)”). The following primers were used: for IPMS1: forward, 5′-GGGCTAGCATG-GAAAGATTACGTACGG-3′ (Nhel); reverse, 5′-GGGGCCGGCCGCTGATTTACAACCGGTTC-3′ (NotI); for IPMS2: forward, 5′-CCAAATTCATGAAACCAAAACCCATTCC-3′ (Ndel); reverse, 5′-GGCAAGCTTCTACTGATAGGTGGATG-3′ (HindIII).

These gene fragments were digested by Nhel/NotI or Ndel/HindIII and inserted into the pET-28b expression plasmid, resulting in N-terminal hexahistidine (His6)-tagged pET-28b-LbIPMS1/2. The plasmid was transformed into Escherichia coli BL21(DE3) strain (Novagen), and the transformed bacterial cells were cultured at 37 °C in LB medium containing 50 μg/ml kanamycin.

Protein expression was induced by adding isopropyl β-D-thiogalactoside into the medium to a final concentration of 1 mM. The cells were harvested by centrifugation at 5000 × g for 10 min at 4 °C, resuspended in a lysis buffer (50 mM Tris-HCl (pH 8.0), 300 mM KCl, and 1 mM PMSF), and then disrupted using a French press. The recombinant protein was purified with affinity chromatography using a Ni2+-nitrilotriacetic Superflow column (Qiagen) pre-equilibrated with buffer A (50 mM Tris-HCl (pH 8.0) and 300 mM KCl) and then washed with buffer B (buffer A supplemented with 10 mM imidazole for IPMS2 or 40 mM imidazole for IPMS1) to remove nonspecifically bound proteins. The target protein was eluted with buffer C (buffer A supplemented with 100 mM imidazole for IPMS2 or 300 mM imidazole for IPMS1), and the eluted fractions were dialyzed against buffer D (20 mM Tris-HCl (pH 8.4) and 50 mM KCl). After purification, the target protein was of sufficient purity (above 95%) and was then concentrated to approximately 10 mg/ml in buffer D by ultracentrifugation for further structural and biochemical studies.

Selenomethionine-substituted LbIPMS2 suitable for structure determination was prepared following the method described previously (17). Purification of the selenomethionine LbIPMS2 protein was performed using the same methods as for the native protein. Expression and purification of the LbIPMS2 truncations were the same as for the full-length protein as described above.

Crystallization and Diffraction Data Collection—Purified LbIPMS2 protein was used for the crystallization experiments, which were performed at 4 °C using the hanging drop vapor diffusion method. Crystals were grown in a drop containing equal volumes (1 μl) of the protein solution (5 mg/ml) and the reservoir solution (0.1 M Tris-HCl (pH 8.5), 0.8 mM sodium formate, and 30% PEG 2000 monomethyl ether). The crystals of LbIPMS2 were later found to contain the substrate α-Kiv; therefore this structure represents the protein complexed with the substrate α-Kiv (LbIPMS2-Kiv). The LbIPMS2-IPM complex crystals were prepared by co-crystallization with substrates α-Kiv, Zn2+, and acetyl-CoA. Attempts to crystalize the LbIPMS2 in the presence of acetyl-CoA have been unsuccessful so far.

For diffraction data collection, the LbIPMS2 crystals were first cryoprotected using Paratone oil (Hampton Research) and then flash cooled in liquid nitrogen. Selenium single wavelength anomalous dispersion diffraction data of LbIPMS2 were collected to a resolution of 2.9 Å from a flash cooled crystal at 100 K at the Photon Factory (Japan), beamline BL5A. The native diffraction data were collected to a resolution of 2.0 Å at beamline NW12. LbIPMS2-IPM was collected to 2.2 Å at beamline BL17U of Shanghai Synchrotron Radiation Facility.
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(China). All the diffraction data were processed, integrated, and scaled together using the HKL2000 suite (18). The crystals of native LbIPMS2 belong to space group $P3_121$, containing one LbIPMS2 molecule in the asymmetric unit with a solvent content of 60%. The statistics of the diffraction data are summarized in Table 2.

Structure Determination and Refinement—The structure of the LbIPMS2-Kiv complex was solved using the single wavelength anomalous dispersion method implemented in the program SOLVE (19). The single wavelength anomalous dispersion phases were improved by statistical density modification, including solvent flattening and histogram matching, using the program RESOLVE (20), increasing the overall figure of merit from 0.39 to 0.77 at 2.9-Å resolution. The resultant electron density map was of high quality, and RESOLVE automatically built 60% of the polyalanine model. The full-structure model was built manually using the program Coot (21). Structure refinement was carried out against the 2.0-Å native data using the program PHENIX with standard protocols (22). The structure of the LbIPMS2-IPM complex was solved by molecular replacement using PHENIX with the structure of the LbIPMS2-Kiv complex used as the starting model. In the structures, there is strong electron density at the active site that matched the bound substrate or product very well. In addition, there was a residual electron density near the bound substrate that could be fitted with a divalent metal ion. The statistics of the structure refinement and the quality of the structure models are summarized in Table 2.

Enzymatic Activity Assay—The enzymatic activity of both wild-type and mutant LbIPMS1/2 were assayed by monitoring the production of reduced CoA (HS-CoA) over time as wild-type and mutant refined and the quality of the structure models are summarized in Table 2.

The optimal pH for LbIPMS1/2-catalyzed reactions was determined in the pH range of 6.5–10.1 in about 0.4-pH unit increments under conditions otherwise the same as that of standard, but the Tris-HCl buffer (0.1 M) in reaction assays was replaced by MES (pH 6.5), HEPES (pH 6.5–8.1), Tris-HCl (pH 8.1–8.9), or glycine (pH 8.9–10.1) buffer (0.1 M in all cases). Because an excess amount of divalent (2 mM Mn$^{2+}$) and monovalent ions (50 mM K$^+$) was included in the standard enzymatic assay systems, a further ionic strength change caused by pH adjustment may be avoided. The optimal reaction temperature for LbIPMS1 and LbIPMS2 was determined in the reaction temperature range of 0, 20, 37, 45, 50, 60, and 65 °C. The thermostability assay was performed by treating LbIPMS1/2 at different temperatures of 0, 20, 30, 37, 40, 45, 50, 55, 60, and 65 °C for 30 min before cooling on ice. The above treated enzyme was then used in the catalytic reaction.

In the experiments to measure the effects of different metal ions on the enzymatic activity of LbIPMS1/2, we removed the bound metal ions co-purified with the enzyme by using a reported protocol (11). Then the protein was used for the activity assay in the presence of different metal ions.

Complementation Assay—A complementation assay was carried out in E. coli CV512 strain, which lacks functional IPMS. The strain is able to grow on M9 medium only when transformed with functional IPMS. The full length and truncations of LbIPMS1/2 cDNA were amplified and linked with the plasmid pTRC99a. The resulting new plasmid was transformed into E. coli CV512, and the empty vector pTRC99a was used as a negative control. The strain was cultured under 37 °C on an M9 medium plate supplemented with 1 mM isopropyl β-D-thiogalactoside.

RESULTS

LbIPMS2 Exhibits Catalytic Activities Comparable with That of LbIPMS1 with a Unique Characteristic of Unresponsiveness to Monovalent Cation Activation—Similar to L. interrogans, the genome of L. biflexa also bears three copies of leuA-like genes. LEBL_11291, which shares high amino acid sequence similarity to cimA of L. interrogans (LA_2350), is likely to encode a CMS. LEBL_11845 and LEBL_11108 are most likely to encode IPMSs based on their high sequence similarities to their counterparts in L. interrogans, LA_2202 (leuA1) and LA_0469 (leuA2), respectively (24). The 500-residue protein encoded by LEBL_11845 (leuA1) is designated as LbIPMS1, and the 394-residue protein encoded by LEBL_11108 (leuA2) is designated as LbIPMS2. Sequence analyses show that LbIPMS1 has a structure arrangement similar to that of MtIPMS and is correspondingly composed of an N-terminal domain, a C-terminal regulatory domain, and two connecting subdomains I and II, whereas LbIPMS2 contains only the N-terminal domain and the connecting subdomains, apparently lacking the C-terminal regulatory domain (Fig. 1).
The two leuA genes from *L. biflexa* were individually expressed in *E. coli*, and the recombinant proteins were affinity chromatographically purified to /H1102290% as detected by SDS-PAGE. The enzymatic activities indicate that, in addition to the natural substrate /H9251-Kiv, both *Lb* IPMS1 and *Lb* IPMS2 can catalyze the condensation reaction of transferring the acyl group from acetyl-CoA to several /H9251-keto acids, and *Lb* IPMS1 shows a broader substrate spectrum than *Lb* IPMS2. It is noteworthy that the *Km* values of *Lb* IPMS2 for both acetyl-CoA and /H9251-Kiv are 2-fold higher than those of *Lb* IPMS1, leading to an approximately 2-fold decrease of the catalytic efficiency (Table 1).

**Activation of IPMS by monovalent cations** is well documented for the enzymes from several organisms, including the activation of *Mt* IPMS by K⁺ (23, 25–28). Additional divalent metal ions cannot activate the enzymes from yeast, *Salmonella typhimurium*, or *Alcaligenes eutrophus* (23, 27–29) but are able to further improve the activities of both *Mt* IPMS (14, 26) and *Li* CMS (11). In this study, we demonstrated that the activities of both *Lb* IPMS1 and *Lb* IPMS2 can be potentiated by several divalent metals with Mn²⁺ as the most effective activator followed by Mg²⁺, Co²⁺, and Ca²⁺, whereas Zn²⁺ may slightly inhibit the activities of both enzymes (Fig. 2A). The activation of *Lb* IPMS1 and *Lb* IPMS2 by Mn²⁺ was concentration-dependent with the maximum at 0.5 mM Mn²⁺ (Fig. 2B). However, it was unexpected that although the activity of *Lb* IPMS1 was co-activated by monovalent metal ions with K⁺/NH₄⁺ being the

![Figure 1. Structure-based sequence alignment of representative isopropylmalate synthase/IPMS-like Claisen enzymes.](image-url)
most effective in the presence of Mn$^{2+}$ (*i.e.* 20 mM K$^+$ was required for the maximum effect) the activity of *Lb*IPMS2 was not affected by any of the monovalent metal ions examined (Fig. 2, C and D). This finding suggested a speculative association with the short form structure of *Lb*IPMS2 among all of the IPMSs studied so far. However, we failed to identify any of its other biochemical properties that might account for or be caused by this unique character. Nevertheless, to keep the assay conditions consistent, 50 mM K$^+$ and 2 mM Mn$^{2+}$ were added in the reaction systems for both enzymes throughout the study (“Experimental Procedures”).

The optimum pH values for both *Lb*IPMS1 and *Lb*IPMS2, measured as $V_{\text{max}}$ values, were 8.5. This result is highly similar to that previously characterized for IPMSs from *M. tuberculosis* (10), *S. typhimurium* (23), *A. eutrophus* (29), and plant, *i.e.* the two isoforms from *Arabidopsis* (30). The optimum reaction temperature for *Lb*IPMS2 (50°C) was much higher than that for *Lb*IPMS1 (37°C) (Fig. 2E). The stability of *Lb*IPMS2 was remarkably decreased when the temperature exceeded 45°C and was higher than that of *Lb*IPMS1at 37°C. Therefore, the enzymatic assay was carried out at 45°C with *Lb*IPMS2 and 37°C with *Lb*IPMS1.

**Crystal Structure of *Lb*IPMS2 Reveals the Active Center for Binding the Substrate and the Product—**We solved the crystal structure of *Lb*IPMS2 by the single wavelength anomalous dispersion method (“Experimental Procedures”). Each asymmetric unit contains one *Lb*IPMS2 molecule bound with the substrate α-Kiv and a Zn$^{2+}$ at the active center, representing the substrate complex (*Lb*IPMS2-Kiv). As we did not add α-Kiv during protein purification or crystallization, the substrate should be acquired from the host *E. coli*. Acetyl-CoA and Zn$^{2+}$ were added in other co-crystallization trials, and the final electron density map showed a good match for the reaction product α-IPM, representing the product complex (*Lb*IPMS2-IPM). The statistics of the final structure models are summarized in Table 2. As the two structures do not show significant conformational differences, we used the structure of *Lb*IPMS2-Kiv as the representative for description.

The structure of *Lb*IPMS2-Kiv contains residues 3–301 and 310–387 with residues 302–309 disordered. Similar to *Mt*IPMS, the overall structure of *Lb*IPMS2 can be divided into three parts: the N-terminal domain, subdomain I, and subdomain II (Fig. 3A). The N-terminal domain (residues 3–289) adopts a (β/α)$_8$ TIM barrel fold with α-Kiv and Zn$^{2+}$ bound at the active center located in the middle of the barrel. Subdomain I (residues 290–333) forms an α-helix (α10), a short 3$_{10}$ helix, and two loops packing against α10. Subdomain II (residues 334–394) consists of three α-helices, α11, α12, and α13, which form a compact helical bundle mediated by hydrophobic residues Ile-343/Leu-346/Leu-347 of α11, Ile-359/Leu-362/Phex-363/Ala-366 of α12, Leu-383/Val-384/Leu-386 of α13, and Phe-334/Ile-352/Val-354/Ile-378 of the connected loops. Two types of interactions (type A and type B) between the symmetry-related molecules were identified. The type A interactions were formed between the N-terminal domains and buried 4709 Å$^2$ (30.2%) of the total surface area. Similar types of interactions have also been identified in the structures of *Mt*IPMS and LiCMSN, suggesting that the dimerization of *Lb*IPMS2 is biologically relevant (Fig. 3B), and this was supported by size exclusion chromatography of the active enzyme in solution (data not shown). The type B interactions were formed between two subdomains II via mainly hydrogen bonds and buried 1785 Å$^2$ (11.3%) of solvent-accessible surface area (Fig. 3C).

At the active center of *Lb*IPMS2, the substrate α-Kiv is stabilized by a metal ion. In the structures of LiCMSN and *Mt*IPMS, a Zn$^{2+}$ ion was identified to bind at the same site (9, 11). As the bound metal ion in *Lb*IPMS2 is coordinated by six ligands (His-209, 2.3 Å; His-207, 2.2 Å; Asp-15, 2.1 Å; water 14, 2.3 Å; and two carbonyl oxygens of α-Kiv, both 2.3 Å) with an octahedral geometry in a similar way as the Zn$^{2+}$ ion in the structures of LiCMSN and *Mt*IPMS, we speculate that it is very likely a Zn$^{2+}$ ion as well even though we did not determine it chemically. The substrate α-Kiv also makes hydrogen-bonding interactions with two residues, Thr-178 and Arg-14 (2.3 and 2.9 Å, respectively) (Fig. 4A). All of these residues are highly conserved among IPMSs from different species (Fig. 1), suggesting a common binding mode of the substrate. When the product is formed as the result of the catalytic reaction, as shown by the structure of the *Lb*IPMS2-IPM complex, the conformation of *Lb*IPMS2 does not show much difference from the *Lb*IPMS2-Kiv complex (root mean square deviations are 0.6 Å). In the structure of *Lb*IPMS2-IPM, a metal ion was also found to bind at the active center with similar coordination geometry, and

**TABLE 1**

Kinetic data for the catalytic reaction of the *Lb*IPMS1/2

All kinetic parameters are the means of duplicate determinations. ND, undetectable, the enzymatic activity was too low to be detected; Kb, α-ketobutyrate; Kiv, α-ketoisovalerate; K2, oxovalerate; Ks, 4-methylthio-2-oxobutyrate; Kh, 2-oxohexanoate.

|            | **LbIPMS1** |            | **LbIPMS2** |
|------------|-------------|------------|-------------|
|            | $K_m$ (μM)  | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ (s$^{-1}$ μM$^{-1}$) | $K_m$ (μM)  | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ (s$^{-1}$ μM$^{-1}$) |
| Fixed acetyl-CoA, variable ketoacid |            |            |            |            |            |            |
| Kb         | 732.8 ± 62.7 | 6.5 ± 0.2  | 8.9 ± 0.8 × 10$^2$ | 1374.0 ± 40.7 | 2.8 ± 0.02 | 2.0 ± 0.06 × 10$^3$ |
| Kiv        | 269.2 ± 31.3 | 2.5 ± 0.05 | 9.1 ± 1.0 × 10$^2$ | 598.4 ± 12.5 | 2.4 ± 0.009 | 4.0 ± 0.10 × 10$^2$ |
| K2         | 245.5 ± 49.3 | 1.1 ± 0.05 | 4.5 ± 0.9 × 10$^2$ | 314.8 ± 22.3 | 0.14 ± 0.0004 | 4.6 ± 0.5 × 10$^2$ |
| Ks         | 363.0 ± 6.1  | 0.2 ± 0.001| 6.4 ± 0.1 × 10$^2$ | ND           | ND           | ND           |
| Kh         | 185.9 ± 8.4  | 0.08 ± 0.001| 4.2 ± 0.07 × 10$^3$ | ND           | ND           | ND           |
| Fixed ketocids, variable acetyl-CoA |            |            |            |            |            |            |
| Kb         | 520.0 ± 19.7 | 3.2 ± 0.04 | 6.1 ± 0.2 × 10$^2$ | 1078.9 ± 151.5| 2.2 ± 0.06 | 2.0 ± 0.4 × 10$^3$ |
| Kiv        | 659.2 ± 83.0 | 2.7 ± 0.04 | 4.1 ± 0.5 × 10$^2$ | 1164.1 ± 44.2| 2.0 ± 0.02 | 1.8 ± 0.07 × 10$^2$ |
| K2         | 685.5 ± 71.3 | 1.5 ± 0.02 | 2.2 ± 0.2 × 10$^2$ | 6180.0 ± 51.0| 0.2 ± 0.002 | 2.6 ± 0.11 × 10$^2$ |
| Ks         | 779.8 ± 33.8 | 0.3 ± 0.01 | 3.7 ± 0.2 × 10$^2$ | ND           | ND           | ND           |
| Kh         | 6668.1 ± 713.0| 0.3 ± 0.04 | 3.8 ± 0.8 × 10$^2$ | ND           | ND           | ND           |
most of the residues constituting the active center assume similar positions and conformations as observed in the structure of LbIPMS2-Kiv. However, the side-chain conformation of Gln-18 in LbIPMS2-IPM rotates about 180° compared with that in the structure of LbIPMS2-Kiv and forms two hydrogen bonds with the carbonyl oxygen of α-IPM; one is direct, whereas the other is via a water molecule (water 123). In addition, water 14 also forms a hydrogen bond (2.4 Å) with the carbonyl oxygen of α-IPM (Fig. 4B). In general, the structures of the enzyme bound with either the substrate or the product are highly similar to each other, albeit we cannot exclude the possibility of potential conformational changes during the reaction process, especially for acetyl-CoA binding.

Intact Subdomain II Is Required for the Catalytic Activity of Both Short Form and Long Form LbIPMSs—Because LbIPMS2, which lacks the regulatory domain, has enzymatic activity comparable with that of LbIPMS1, we hypothesized that the regulatory domain might not be required for the activity of LbIPMS.

To evaluate this hypothesis, we truncated the regulatory domain of LbIPMS1 at residue Arg-385 (LbIPMS1-R385) (hereafter, the truncation mutant is named as “protein-truncated residue” for short) to construct an LbIPMS2-mimetic protein based on the structure-based sequence alignment (Fig. 1). The enzymatic assay showed that LbIPMS1-R385 retained 90% of the activity of the full-length LbIPMS1 (Fig. 5A). The activation of LbIPMS1-R385 by di- or monovalent metals also showed patterns similar to that of the full-length LbIPMS1 (Fig. 2). The activation of LbIPMS1-R385 by di- or monovalent metals also showed patterns similar to that of the full-length LbIPMS1 (Fig. 2). These results confirm our hypothesis that the presence of the regulatory domain is not required for the enzymatic activity and further suggest that activation of LbIPMS1 by monovalent metals is independent of the regulatory domain.
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**Table 2**
Summary of diffraction data and structure refinement statistics

|                          | Se-Met | LbIPMS2 | LbIPMS2-Kiv | LbIPMS2-IPM |
|--------------------------|--------|---------|-------------|-------------|
| **Statistics of diffraction data** |        |         |             |             |
| Beamline                  | BL5A   | NW12    | BL17U       |             |
| Wavelength (Å)            | 0.9790 | 1.0000  | 0.9795      |             |
| Resolution range (Å)     | 50.0–2.9 | 50.0–2.0 | 50.0–2.2    |             |
| r.m.s. (Å)               | (3.0–2.90) | (2.07–2.00) | (2.28–2.20) |             |
| Space group              | P31,21 | P31,21  | P31,21      |             |
| Cell parameters          | a (Å)  | 129.5   | 130.0       | 131.4       |
|                          | b (Å)  | 129.5   | 130.0       | 131.4       |
|                          | c (Å)  | 46.5    | 46.7        | 46.7        |
|                          | b′ (%) | 90.0    | 90.0        | 90.0        |
|                          | b′ (%) | 90.0    | 90.0        | 90.0        |
| Average redundancy       | 27.0 (22.2) | 10.5 (9.9) | 6.2 (5.5)   |
| Average r(f/σ(r))        | 36.9 (2.2) | 42.7 (4.2) | 14.5 (3.1)  |
| Completeness (%)         | 99.5 (97.8) | 99.9 (100.0) | 100.0 (99.9) |
| r_{merge}%               | 13.5 (45.7) | 6.3 (47.2) | 12.2 (41.8) |

**Statistics of refinement**

|                           |        |         |             |             |
| R_{merge}/R_{free} (%)    | 15.9/20.9 | 16.1/19.3 |             |             |
| Total protein atoms       | 2,933  | 2,953   |             |             |
| Total ligand atoms        | 9      | 13      |             |             |
| Total solvent atoms       | 263    | 148     |             |             |
| Average B factor (Å²)     | 39.2   | 29.5    |             |             |
| Protein                   | 36.8   |         |             |             |
| Kiv                       | 40.9   | 23.7    |             |             |
| IPM                       | 43.1   | 28.4    |             |             |
| r.m.s. bond lengths (Å)   | 0.007  | 0.007   |             |             |
| r.m.s. bond angles (%)    | 1.1    | 1.2     |             |             |
| Ramachandran plot (%)     | 97.1   | 96.3    |             |             |
| Favored regions           | 100.0  | 100.0   |             |             |
| Allowed regions           |        |         |             |             |

Numbers in parentheses represent the highest resolution shell.

|                           |        |         |             |             |
| R_{merge} = ∑_{i=1}^{N} F_{i}(hkl) − ∑_{i=1}^{N} F_{i}(hkl) / ∑_{i=1}^{N} F_{i}(hkl).
| R = ∑_{i=1}^{N} F_{i} − | F_{i} | ∑_{i=1}^{N} F_{i}.

The crystal structure of LbIPMS2 shows a novel organization such that subdomain II is positioned away from the N-terminal domain of the same molecule and close to the active center of the adjacent molecule in the biologically relevant dimer (Fig. 3B). To clarify the functional role of subdomain II in the enzymatic activity, we further truncated LbIPMS2 from its C terminus in different lengths and measured the enzymatic activities of the resultant mutants (Fig. 5A). The results show that either removal or disruption of subdomain II could lead to complete loss or a dramatic decrease of the enzymatic activity, and the minimum length required for the activity is LbIPMS2-S389, which retains about 64% activity of the full-length LbIPMS2. Further deletion of more residues either almost disrupted the activity (i.e. LbIPMS2-S387; 0.3% activity remained) or completely abolished the activity (i.e. LbIPMS2-R376). These data are consistent with the in vivo complementation assay results. The leuA− mutant containing LbIPMS2-S389 grew normally; in contrast, that containing the LbIPMS2-S387 grew very slowly, and that containing LbIPMS2-R376 did not grow at all (Fig. 5A). In the structure of LbIPMS2, Ser-389 is located at the C terminus of α13, which is on the distal side of the active center of the adjacent monomer and packs against α11 and α12 through hydrophobic interactions (Figs. 1 and 3C). LbIPMS2-S387 (i.e. removing the C-terminal residues of α13) or LbIPMS2-R376 (i.e. complete deletion of α13) would expose the hydrophobic core of subdomain II to the solvent and therefore destabilize the overall structure of subdomain II (Fig. 3D).

Consistently, these two LbIPMS2 truncation mutants purified from the heterogeneous expression system seemed unstable (Fig. 5B). To verify these observations, we generated several equivalent truncation mutants of LbIPMS1 based on the sequence alignment with LbIPMS2. Enzymatic activity assays and genetic complementation phenotypes of these mutants showed similar results as the LbIPMS2 mutants, strongly suggesting that an intact subdomain II is required for the enzymatic activity of LbIPMS2 and LbIPMS1 (Figs. 1 and 5A).

**Subdomain II Is Likely Involved in the Conformation Transition during Catalysis That Might Be Associated with the Binding to Acetyl-CoA**—In the dimeric MtiIPMS, the only known structure for the long form IPMSs, the two monomers exhibit significant conformational differences. In particular, subdomain II of monomer A is located on the top of the active site of monomer B, whereas subdomain II of monomer B is far away from the active center of monomer A (9, 31). In contrast, in the short form dimeric LbIPMS2, two symmetry-related monomers form a homodimer (Fig. 6), and the conformation of both subdomains II are similar to that of monomer A but substantially different from that of monomer B in MtiIPMS (Fig. 6). Because of the flexibility of the linker between subdomains I and II, it is reasonable to hypothesize that subdomain II may adopt different conformations, and one of them should represent the state during the catalytic reaction.

Full-length LbIPMSs may be involved in two kinds of allosteric conformational changes during catalysis. One is the homotropic positive cooperation between the two monomers with either of the two substrates as an effector. A particularly complex example of this kind of allostery is found in the IPMS from *A. eutrophus* H16. With its Hill coefficient for either of the two substrates alternating between values higher and lower than 1.0, the cooperativity of the enzyme seems to change between positive and none along with variation of the concentration of the substrates (32). Another form is heterotropic inhibition caused by the binding of the ligand, for instance l-leucine for IPMSs of many different origins (1, 23, 33). The homotrophic positive cooperative reaction features of LbIPMS2 and its homolog LbIPMS1-R385 (Fig. 7) implicate that the two monomers of the functional dimer may adopt different conformations corresponding to different catalytic states with or without the substrates bound. The Hill numbers of these enzymes indicated that the positive cooperativity against acetyl-CoA was stronger (or more complex; for n_{h1} > 2) than that against α-Kiv (n_{h1} = 1.8) (Fig. 7). In fact, similar positive cooperativity was found in the long form LbIPMS1 as well (derived from data in Fig. 8), but the Hill number for either of the two substrates (1.6–1.8) was lower than that of the short form enzymes.

The questions of whether and how subdomain II is related to this allosteric effect was further studied. Among the series of truncation mutants of LbIPMS2, LbIPMS2-Q353, which contains only a small portion (helix α11) of subdomain II (Fig. 1), is special because it could not catalyze the catalytic reaction for α-Kiv but retained the ability to catalyze the condensation reaction of ketobutyrate with very low activity (more than a 97% decrease of the k_{cat}). However, its k_{m} toward ketobutyrate was...
**FIGURE 3.** The crystal structure of LbIPMS2. A, overall structure of LbIPMS2 in complex with α-Kiv. B, the homodimer structure of LbIPMS2. C, subdomain II forms a homodimer with another symmetry-related subdomain II; the interactions mainly involve hydrogen bonds (shown with dashed lines). D, surface model of subdomain II showing the hydrophobic core and the location of α13. Mol, molecule; Wat, water.

**FIGURE 4.** Stick model of the active center of LbIPMS2. A, the binding site of the substrate α-Kiv. B, the binding site of the product α-IPM. The residues forming the binding site are shown with green sticks. The substrate α-Kiv and the product α-IPM are shown with magenta sticks, and the divalent metal ions and water (W) molecules are shown with gold and red spheres, respectively. The interactions are indicated with dashed red lines.
nearly unaffected (Table 3), consistent with the previous report that deletion of the regulatory domain of MtIPMS did not affect the α-Kiv binding (16). Conversely, the $K_{m}$ of this truncated enzyme for the cofactor acetyl-CoA decreased by 2-fold (Table 3). This result implies that, as an essential part of the intact catalytic unit, subdomain II might be functioning in the recruitment and binding of acetyl-CoA.

We also investigated the feedback inhibition for the full-length LbIPMS1. Similar to that observed for MtIPMS (1), a V-type inhibition was shown in the case of LbIPMS1 against the substrate α-Kiv with its $K_{m}$ value almost unchanged (Fig. 8C). However, when the inhibitory effect against acetyl-CoA was measured for LbIPMS1 in the presence of 1 mM l-leucine, the $k_{cat}$ was about 68.8% of that of the apoenzyme, indicating a V-type inhibition. Meanwhile, the $K_{m}$ was about twice of that of the apoenzyme, inferring a K-type inhibition mechanism (Fig. 8B). Therefore, the inhibitory effect against substrate acetyl-CoA is complex, but a mechanism involving acetyl-CoA binding in the process of feedback inhibition might be inferred to the first order of approximation (Fig. 8).

**FIGURE 5.** The activity and stability of the truncation mutants of IPMSs. A, schematic diagrams of different truncation mutants of IPMSs showing their in vitro catalytic activities and in vivo growth complementation phenotypes. Truncation mutants from our experiments are shown in light blue, and the truncation mutants from the literature are shown in light orange. (The activity of truncation mutants of LbIPMS1/2 were experimentally tested, and the relative activity was calculated based on the specific activity of the wild-type LbIPMS2 (2.8 × 10^3 nmol/mg min), which was 100. a, not detected (ND); b, undetectable (ND) (i.e. the specific activity is less than 0.1% of that of LbIPMS2); c, reported undetectable; d, for MtIPMS-F457, which retains 12% of the activity of full-length MtIPMS (100%) (14). The clones expressing truncations of LbIPMS1 and LbIPMS2 were transformed into a leuA-null mutant to observe their capability of complementing the auxotrophic growth defect of the host. +, growth similarly to the full length; +, growth slower than the full length; --, cannot grow. B, the truncation mutants were expressed heterologously and purified, and the purity and stability of the resulting proteins were checked by SDS-PAGE. M, molecular mass markers.

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In this study, we characterized structurally and biochemically the natural short form \textit{LbIPMS2} from \textit{L. biflexa} that lacks the regulatory domain but has catalytic activity comparable with that of the long form \textit{LbIPMS1} from the same species. Based on these analyses, we further tested a series of truncation mutants from the C terminus of subdomain II of \textit{LbIPMS2} to pinpoint the minimal structural components essential for catalysis (Fig. 5A). The results indicate that either removal or disruption of subdomain II may lead to the complete loss or a dramatic decrease of the enzyme activities, whereas deletions that left the subdomain II unaltered had little effect upon the activities. Therefore, we conclude that the catalytic activity of IPMS is independent of its regulatory domain but requires an intact subdomain II. In other words, the previously defined “N-terminal catalytic domain” in \textit{MitIPMS} and \textit{LiCMSN} (9, 11, 12) is actually incomplete, and by integrating the N-terminal domain hosting the catalytic center with the complete subdomain I and subdomain II, a catalytic module, which is functionally and structurally independent of the regulatory domain, is defined.

This conclusion appears to conflict with the results of previous mutation analyses conducted in IPMS/CMSs regarding the role of the regulatory domain in maintaining the full enzymatic activity. In the case of \textit{Saccharomyces cerevisiae} IPMS (ScIPMS), an “R” region (514–552) deletion leads to complete loss of the ScIPMS activity. Because this R region is likely located in the middle of the regulatory domain corresponding to the structure of \textit{MitIPMS} (Fig. 1), this result echoes our data, at least to a certain extent, that partial truncation of the regulatory domain would cause significant, albeit not necessarily complete, loss of the \textit{LiCMS} activity (Fig. 5A). In the other two typical studies (15, 16), truncation of the C-terminal regulatory domain in both IPMSs from \textit{Neisseria meningitidis} and \textit{M. tuberculosis} is shown to result in the complete loss of catalytic function with limited structural changes to the active sites of either of the truncated proteins observed (16). However, structure-based sequence alignment matching the C-terminal residues of those truncations, \textit{i.e.} the \textit{NisIPMS-E365} and \textit{MitIPMS-V425}, to \textit{LbIPMS2} residues, \textit{i.e.} the Ser-368 and Thr-331, respectively, indicated that not only removal of the regulatory domain but also disruption of subdomain II can occur (Figs. 1 and 5A). Therefore, the catalytic module was disrupted in these studies.

By searching the protein structure database, we found that the homocitrate synthase from \textit{Schizosaccharomyces pombe} (\textit{SpHCS}) is another short form Claisen enzyme that catalyzes the condensing reaction of \textit{\alpha}-ketoglutarate and acetyl-CoA. Composed of an N-terminal domain followed by subdomain I and subdomain II, \textit{SpHCS} is not only structurally (Protein Data Bank code 3IVS) (34) similar to \textit{LbIPMS2}, but its subdomain II has been shown to be essential for catalytic activity (35). Therefore, together with the structure/function characteristics of \textit{SpHCS}, the basic catalytic module defined in this study, using \textit{LbIPMS2} as a representative, should be regarded as a general model for the IPMS-like Claisen enzymes no matter whether an extra regulatory domain is present or not.

The essential function of an intact subdomain II with regard to the catalytic activity of IPMS is somewhat unexpected given the distance from it to the catalytic center. In our previous work, the structure of \textit{LiCMSN} complexed with its substrates (pyruvate and acetyl-CoA) indicates that acetyl-CoA is bound in the deep surface groove of the TIM barrel near the bound pyruvate and is stabilized through Phe-83, Arg-16, Gln-20, and Glu-146 (11), all located on one side of the active center, which does not seem sufficient for stable binding of acetyl-CoA. In this study, we identified that the \( K_w \) of acetyl-CoA was increased about 2-fold in the truncated active enzyme \textit{LbIPMS2-Q353} (Table 3), inferring that subdomain II might be another element involved in stable binding with acetyl-CoA. In addition, the moderate distance of subdomain II to the active center in the symmetric dimer allows us to speculate that the binding of acetyl-CoA may induce the reorientation of subdomain II, which might further adjust the active site residues to the proper position required for catalysis to occur. This speculation is also supported by the homotropic allosteric kinetics property of the IPMSs (23, 32), including \textit{LbIPMS2} (Fig. 7), showing an obvious cooperative effect of substrate binding. We also identified significant difference in the subdomain II orientation between the structure of \textit{LbIPMS2} and the structure of \textit{MitIPMS} in complex with \( \alpha \)-Kiv, respectively (Fig. 6). Because the \textit{MitIPMS} structure with \( \alpha \)-Kiv bound to both monomers was determined via soaking the substrates into the crystal where the preexistent crystal packing might prevent any substrate binding-induced conformational change, the structure may represent the preactive state. Meanwhile, our \textit{LbIPMS2-Kiv} structure may represent the active state because the substrate was co-purified and prebound to the enzyme during crystallization. Therefore, the difference in the orientation of subdomain II between the two structures may reflect the conformational change between the active and preactive states in the widely accepted allosteric model in which it is generally understood that allosteric enzymes in the preactive state can be converted to the active state by substrate binding (3, 36–38).
Comparison of the crystal structure of MtIPMS with or without L-leucine binding revealed no evident structural changes at the active site (9). However, a mechanism of feedback inhibition/allosteric regulation involving interdomain communication via a change in conformational equilibrium rather than changes in a static structure was proposed previously (39, 40) based on the mutation analysis of the critical residue Tyr-410 of MtIPMS subdomain I (Fig. 1; corresponding to Tyr-316 in LbIPMS2) laid over the active site of the adjacent monomer (39). In that study, the Y410F mutant lost feedback inhibition to L-leucine; meanwhile, its \( K_m \) value toward acetyl-CoA decreased about 10-fold, and the \( k_{cat} \) values were reduced by 35-fold relative to that of the wild-type enzyme. Those results suggest that the active site of the Y410F mutant is more accommodable for acetyl-CoA binding, whereas the mutation breaks the pathway for transmitting the signal of L-leucine binding to the catalytic center (39). A similar phenomenon can be found in the mutation analysis of Asp-444 residue located in subdomain II of MtIPMS (corresponding to Arg-350 of LbIPMS2) that also demonstrated higher affinity for acetyl-CoA and less sensitivity to L-leucine feedback inhibition than the wild-type enzyme (31). Considering that the signal of L-leucine binding should pass subdomain II prior to subdomain I from

### FIGURE 7. Cooperative effect of substrate binding

Purified enzymes were used in the assays detailed under “Experimental Procedures.” Enzyme activities were measured against variable concentrations of one substrate while keeping the other in excess. A, Scatchard plots against each of the two substrates binding to LbIPMS2. B, Scatchard plots against each of the two substrates binding to LbIPMS1-R385. Hill numbers against each substrate are shown in the figures.

### FIGURE 8. Feedback inhibition of the enzymatic activity of LbIPMS1 by L-leucine

Purified enzymes were used in the assays detailed under “Experimental Procedures.” Standard deviations (error bars) were determined to access the precision of the data. Hill numbers of the holoenzyme against each of the two substrates were calculated and are given in the text. A, inhibition curve of the enzymatic activity of LbIPMS1 with increased concentration of L-leucine. B, allosteric sigmoidal plot of acetyl-CoA binding to LbIPMS1 at different concentrations of L-leucine. C, allosteric sigmoidal plot of \( \alpha-Kiv \) binding to LbIPMS1 at different concentrations of L-leucine.

### TABLE 3

|            | \( K_m \) (\( \mu \)M) | \( k_{cat} \) (s\(^{-1}\)) | \( k_{cat}/K_m \) (s\(^{-1}\)M\(^{-1}\)) |
|------------|-------------------------|-----------------------------|---------------------------------------|
| **Kb**     |                         |                             |                                       |
| LbIPMS2    | 1374.0 ± 40.7           | 2.8 ± 0.02                  | 2.0 ± 0.06 \times 10^3               |
| LbIPMS2-Q353| 1312.2 ± 45.9           | 0.05 ± 0.1                  | 4.0 ± 0.2 \times 10^3               |
| **Ac-CoA** |                         |                             |                                       |
| LbIPMS2    | 1078.9 ± 151.5          | 2.2 ± 0.06                  | 2.0 ± 0.4 \times 10^3               |
| LbIPMS2-Q353| 2409.9 ± 86.9           | 0.05 ± 0.001                | 2.0 ± 0.09 \times 10^3               |

Comparison of the crystal structure of MtIPMS with or without L-leucine binding revealed no evident structural changes at the active site (9). However, a mechanism of feedback inhibition/allosteric regulation involving interdomain communication via a change in conformational equilibrium rather than changes in a static structure was proposed previously (39, 40)
the regulatory domain, we further hypothesize that subdomain II may have a similar function as subdomain I.

In a deuterium-deuterium exchange assay, three peptides of \(l\)-leucine-bound MtIPMS showed decreased deuterium incorporation compared with the apo-enzyme, indicating that conformational changes may happen in these peptides when \(l\)-leucine binds; i.e. feedback inhibition occurs (40). Structure-based alignment (Fig. 1) mapped two peptides (residues 453–457 and residues 488–495) in subdomain II of \(l\)-leucine-bound MtIPMS to the corresponding residues in subdomain II of \(Lb\)IPMS2, i.e. residues 359–363 and residues 391–394, respectively. The former peptide, located at the second \(\alpha\)-helix of subdomain II adjacent to Gln-353 (this truncation only affects the binding affinity of acetyl-CoA (11), indicating that the conformational changes induced by \(l\)-leucine binding may interfere with binding of acetyl-CoA to these residues. Thus, the inhibition occurring in the active center is most likely achieved through the transition of the signal induced by \(l\)-leucine binding from the regulatory domain to the catalytic module via a certain conformational change of the intermediate domains (subdomain I and subdomain II), which may directly or indirectly be associated with the binding of acetyl-CoA.

This proposed bifunctional role of subdomain II in both acetyl-CoA binding and allosteric transmission between the regulatory domain and the catalytic module is further supported by the significant difference in the orientation of \(Lb\)IPMS2 subdomain II from that of MtIPMS (Fig. 6). This structural difference is likely attributable to the active versus preactive states of the enzyme corresponding to the homotropic allosteric pathway and might be equivalent to the active versus inhibited states of the long form enzyme corresponding to the structural difference. Therefore, an alternative scenario might account for the fact that there is no change observed in the structure with or without the presence of \(l\)-leucine.

Recently, it was shown that V-type inhibition in MtIPMS is due to perturbation of the hydrolysis step by binding of \(l\)-leucine, which has no effect on the \(K_m\) for acetyl-CoA (41). Conversely, the isoleucine feedback inhibition effect on LiCMS is K-type for either pyruvate or acetyl-CoA (11), and acetyl-CoA binding to LiCMS does show low level of cooperativity. In this study, our data about \(Lb\)IPMS1 present a mixed mechanism of feedback inhibition in between that of the other two enzymes with its \(K_m\) and \(k_{cat}\) both affected (Fig. 8). In addition, binding of either of the two substrates demonstrates cooperativity; albeit both are weaker than that of the short form \(Lb\)IPMSs (Fig. 7).

All of these data present a complex spectrum of mechanisms of action in IPMSs and their related enzymes such as LiCMS. Nevertheless, because our data match well with most of the previous biochemical measurements and the truncated \(Lb\)IPMS1-R385 performs with almost identical kinetics as that of \(Lb\)IPMS2 (Fig. 7), we propose that the function of subdomain II might be involved not only in acetyl-CoA binding in the short form IPMS but also in the conformation transition process during feedback inhibition of the long form IPMS along with affecting the binding of acetyl-CoA to a certain extent. This proposed process may be similar to or mimicked by the Y410F mutant of MtIPMS in the absence of \(l\)-leucine in which residues 440–447 and 457–462 in the subdomain II portion of the linker domain of the Y410F enzyme exhibited an increase in deuterium incorporation from exchange (40). However, the structure of long form IPMS in complex with the substrate acetyl-CoA is awaited to disclose the mechanism in detail.

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