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AibA/AibB Induces an Intramolecular Decarboxylation in Isovalerate Biosynthesis by *Myxococcus xanthus*

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Abstract: *Isovaleryl coenzyme A (IV-CoA) is an important precursor for iso-fatty acids and lipids. It acts in the development of myxobacteria, which can produce this compound from acetyl-CoA through alternative IV-CoA biosynthesis (aib). A central reaction of aib is catalyzed by AibA/AibB, which acts as a cofactor-free decarboxylase despite belonging to the family of CoA-transferases. We developed an efficient expression system for AibA/AibB that allowed the determination of high-resolution crystal structures in complex with different ligands. Through mutational studies, we show that an active-site cysteine previously proposed to be involved in decarboxylation is not required for activity. Instead, AibA/AibB seems to induce an intramolecular decarboxylation by binding its substrate in a hydrophobic cavity and forcing it into a bent conformation. Our study opens opportunities for synthetic biology studies, since AibA/AibB may be suitable for the production of isobutene, a precursor of biofuels and chemicals.*

Isovaleryl coenzyme A (IV-CoA) is an essential metabolite in myxobacteria. It is used as a precursor for *iso*-odd fatty acids that maintain membrane fluidity and for cellular signaling during development and fruiting-body formation.[1–5] Typically, IV-CoA is formed by catabolyzing leucine. However, an "alternative IV-CoA biosynthesis" (aib) that generates IV-CoA from three molecules of acetyl-CoA (Ac-CoA) has recently been discovered in *Myxococcus xanthus*.[6] It employs five enzymes from two different operons and is highly active under leucine-limiting conditions.[6] aib starts with the condensation of Ac-CoA with acetoacetyl coenzyme A (AcAc-CoA) catalyzed by the enzyme MvaS,[7] followed by elimination of water by the enzyme LiuC.[8] Subsequent decarboxylation of 3-methylglutaconyl coenzyme A (MG-CoA) is achieved by the heterodimeric enzyme AibA/AibB, resulting in 3,3-dimethylacrylyl coenzyme A (DMA-CoA).[10] In the final reduction step, AibC generates IV-CoA (Figure 1 a).[6,9,10]

Interestingly, while the function of MvaS, LiuC, and AibC can readily be deduced from their sequence, the subunits of the decarboxylase AibA/AibB are related to the α- and β-subunits of CoA-transferases and possess high predicted structural similarity to glutaconyl-CoA transferase from *Acidaminococcus fermentans* (Gct), which catalyzes an unrelated reaction in glutamate fermentation.[10,11] Required for activity of this protein is Glu54, which is highly conserved among other bacterial Gcts.[10,11] In AibA/AibB, this glutamic acid is replaced by cysteine. Mutational studies led to the conclusion that this cysteine is involved in AibA/AibB-catalyzed decarboxylation of MG-CoA, and a cysteine-assisted mechanism was proposed.[10] This would relate AibA/AibB to the CurF ECH2 domain, a cofactor-independent decarboxylase in the biosynthesis of curacin that promotes the decarboxylation of 3-methylglutaconyl acyl carrier protein (MG-ACP) to form 3-methyl-crotonyl ACP (MC-ACP) via a proton relay.[12] However, AibA/AibB and CurF are unrelated and the exact mechanism by which AibA/AibB decarboxylates MG-CoA remained unclear.

We therefore determined crystal structures of AibA/AibB in the apo form and in complex with ligands. To this end, the recombinant production of AibA/AibB was optimized because the previously published procedure was insufficient for structural studies. This required testing numerous expression vectors, since AibB tends to form inclusion bodies. Significantly increased amounts (up to 2.5 mg protein complex/l *E. coli* culture compared to several μg in the previous approach)[10] of soluble AibA/AibB were obtained by fusing AibB to N-terminally His_6-tagged and 3C-protease-cleavable T7-lysozyme as a solubility enhancer while co-expressing with untagged AibA. However, the wildtype protein was recalcitrant to crystallization until two mutations were introduced to decrease surface entropy. The fact that the active center contains a free cysteine allowed easy mercury derivatization

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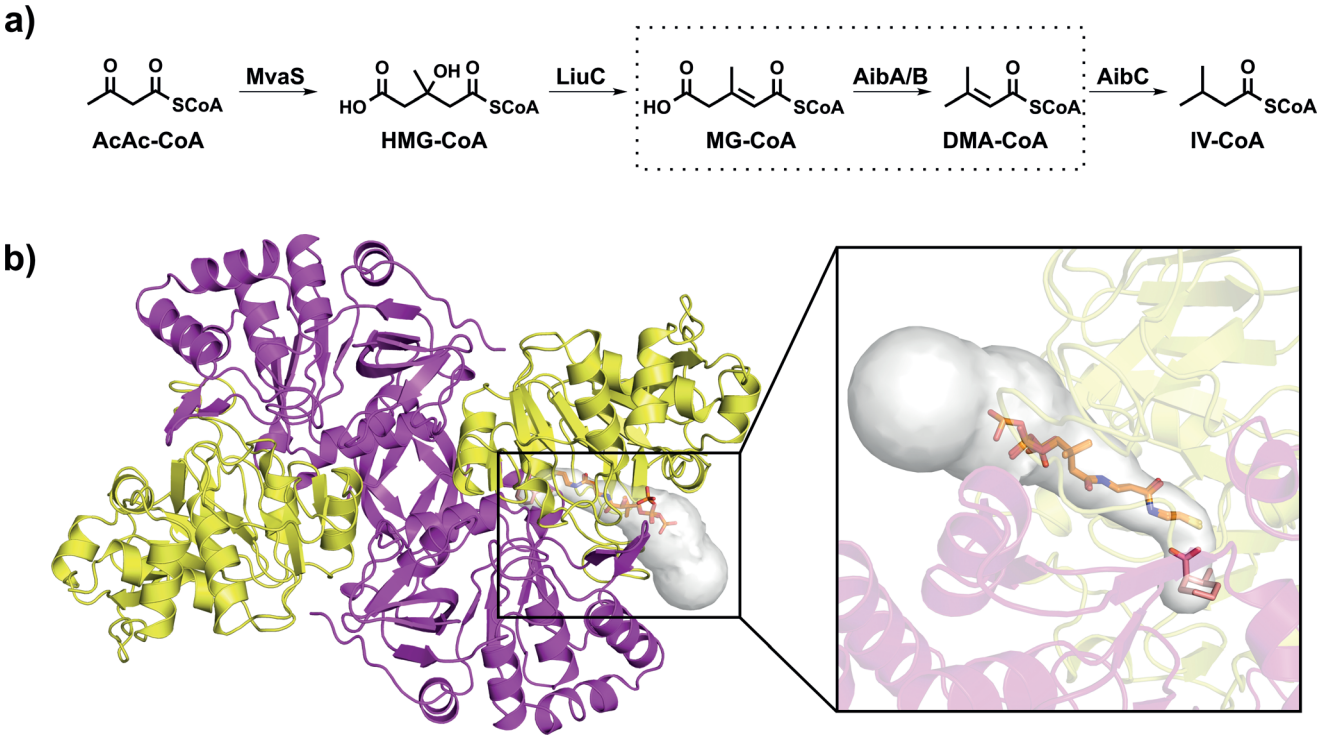

Figure 1. Alternative isovaleryl coenzyme A biosynthesis (aib) and structure of AibA/AibB. a) Overview of aib. The Aib/AibB-catalyzed reaction is highlighted by a dotted box. AcAc-CoA: acetyl coenzyme A, HMG-CoA: 3-hydroxy-3-methylglutaryl-CoA, MG-CoA: 3-methylglutaconyl-CoA, DMA-CoA: 3,3-dimethylacrylyl-CoA, IV-CoA: isovaleryl-CoA. b) Structure of the heterotetrameric AibA/AibB. AibA is shown in magenta and AibB in yellow. The close-up shows the CoA binding site and the active site occupied by a potential transition-state analogue.

and subsequent structure solution from single-wavelength anomalous diffraction data collected at the Hg-L-III-edge.

AibA/AibB crystallized in space group $P2_1$ with two heterodimers in the asymmetric unit. The structure was refined to 1.52 Å resolution with $R_{cryst} = 14.1\,\%$ and $R_{free} = 15.7\,\%$ (Table S3A and S4A in the Supporting Information). Based on the tight packing and an interaction surface of 12110 Å^2 (total surface area 33280 Å^2), the enzyme forms an $(AibA/AibB)_2$ heterotetramer.[13] The alanines introduced to reduce surface entropy ($Glu200Ala^B$, $Glu201Ala^B$) are directly involved in crystal contacts, apparently enabling the formation of well-diffracting crystals (Figure S1 in the Supporting Information).

AibA consists of 11 α-helices and 14 β-strands, whereas AibB is composed of 8 α-helices and 10 β-strands. These secondary structure elements are arranged in a NagB/RpiA/CoA-transferase-like fold characterized by a αβα-layer-sandwich structure (Figure 1b). The two subunits form a complex via polar interactions between at least 14 residues located in the loop regions connecting β3 and β4, β4 and β5, β6 and α5, β7 and α6, and β11 and α8, as well as residues from β4, β6 and α4,5,6,7 of AibA. The interacting residues from AibB are placed in loop regions connecting β3 and α3, and β6 and α5 and residues located in β7, β8 and α3, α4, and α5.

To obtain insight into the decarboxylation mechanism, we attempted to crystallize AibA/AibB in complex with its substrate MG-CoA, which was, however, unsuccessful. Instead, we soaked crystals with saturating amounts of CoA or the substrate acyl group 3-methylglutaconate (3MG) and determined the structures of these complexes at 2.0 Å and 2.3 Å resolution, respectively. The CoA complex showed additional difference electron density (Figure S1A) of nearly complete CoA, but the nucleotide moiety was too flexible to be modeled. Nevertheless, electron density for the pantothenate moiety is clearly visible, showing its binding to the interface of AibA and AibB, which explains why only the complex and not the single monomers is catalytically active. The pantothenate moiety interacts with the protein through direct hydrogen bonds to $Gly104^B$ and $Ser33^B$ and indirectly via two water molecules coordinated by $Gly131^B$ and $Val84^A$ (Figure 2a).

A hydrophobic cavity forms at the end of the active-site funnel, where catalysis is expected to take place. This cavity is built by $Phe27^A$, $Leu53^A$, $Pro54^A$, $Ala74^A$, $Gly106^A$, $Val110^A$, and $Thr181^A$ from AibA, and $Val31^B$, $Ile86^B$, $Leu89^B$, $Pro87^B$, $Phe90^B$, and $Leu138^B$ from AibB. $Cys56^B$, which has previously been speculated to be required for the decarboxylation of MG-CoA, is also part of the active site. Interestingly, this cysteine is the only amino acid with a polar side chain to potentially participate in an acid/base-catalyzed decarboxylation mechanism like the one recently proposed.[10] However, its thiol group is hydrogen-bonded to $Glu72^B$ and points away from the substrate.

While the relatively weak electron density obtained for 3MG points towards a low affinity for this ligand (Figure S2B), the complex indicates that the acyl group of the substrate binds in a bent conformation, which seems to be induced by the constraint space of the active site. The α-

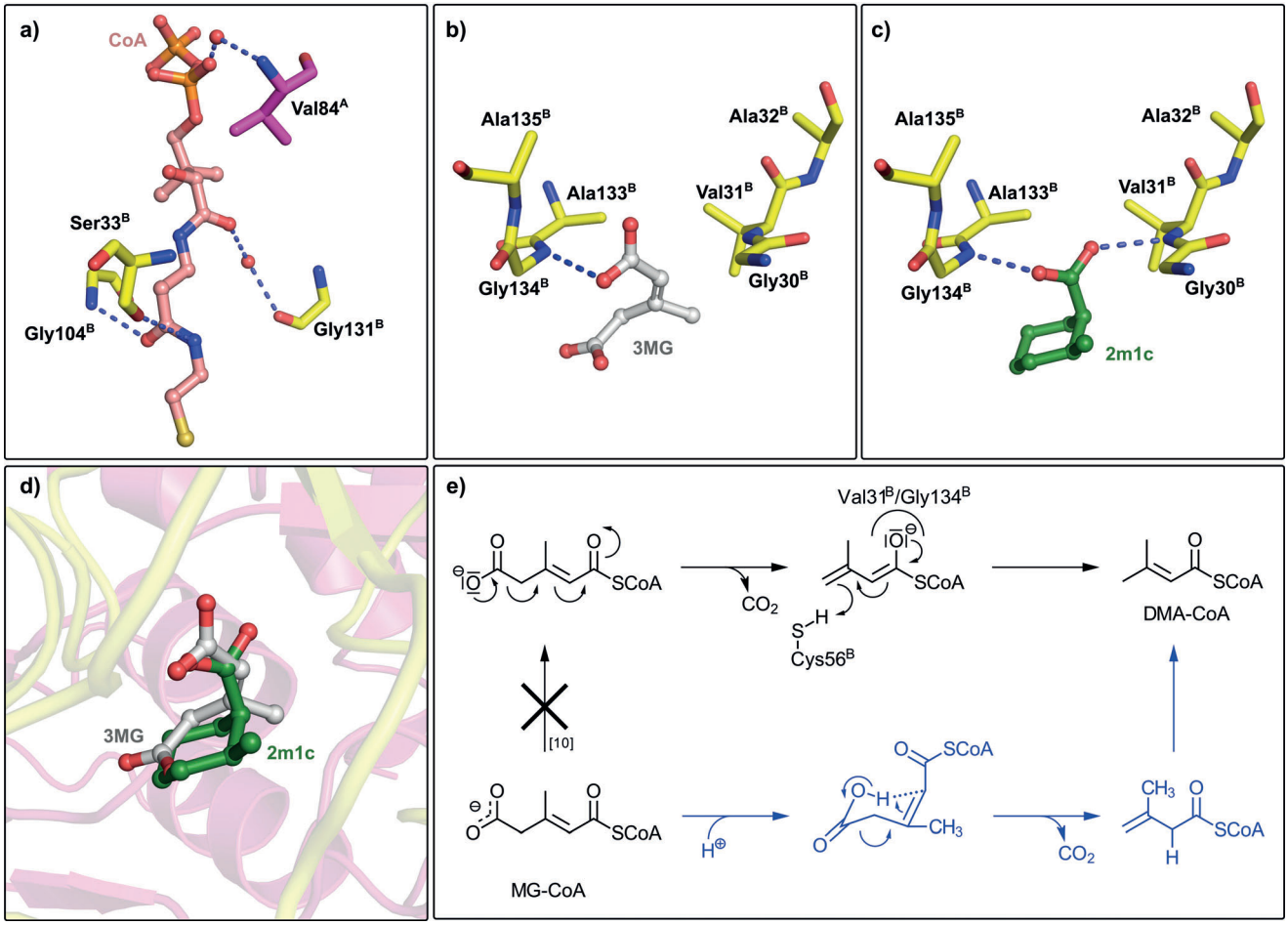

Figure 2. Binding of different ligands and mechanistic hypotheses for the AibA/AibB-catalyzed decarboxylation. a) Fragment of coenzyme A. b) 3-methylglutaconate (3MG). c) 2-methyl-1-cyclohexanecarboxylic acid (2m1c). d) superimposition of 3MG and 2m1c. e) Black: $Cys56^B$-catalyzed decarboxylation as proposed by Li et al.,[10] leading to decarboxylation from a deprotonated γ-carboxylate group. $Cys56^B$ donates a proton to the C-4 position and DMA-CoA is formed. Blue: Intramolecular decarboxylation of the substrate involving the formation of a pericyclic transition state. This mechanism requires a protonated, uncharged γ-carboxylate and hence seems more likely within the hydrophobic active site of AibA/AibB.

carboxylate is involved in a distorted hydrogen bond with the backbone amide of $Gly134^B$, whereas the γ-carboxylate, which will be cleaved off in the course of decarboxylation, resides in a remarkably hydrophobic environment such that it cannot establish any polar contacts with the protein (Figure 2b).

Li and co-workers postulated a $Cys56^B$-assisted decarboxylation mechanism for AibA/AibB, reminiscent of the reaction catalyzed by the unrelated CurF ECH2 (Figure 2e, top).[10] However, this study was performed with expression vectors that yielded only very low amounts of recombinant protein, thus making it possible that the lost activity of the reported $Cys56^B$-Glu/Asp mutants was in fact due to misfolding. We therefore used the expression system established here to mutate $Cys56^B$ to Ala, Ser, Asn, and Val and analyzed the activity and structure of the resulting proteins. The crystal structures of the wild type and the four variants superimpose well, with root mean square (rms) deviations ranging from 0.14 Å to 0.17 Å. To our surprise, all of the variants retained decarboxylation activity and still converted MG-CoA, thus indicating that $Cys56^B$ is not involved in catalysis (Figure S3).

Based on these findings, we propose an enzymatic mechanism in which the substrate catalyzes its own decarboxylation. MG-CoA is an α/β-unsaturated carboxylate with respect to the CoA-thioester, but a β/γ-unsaturated carboxylate with respect to the γ-carboxylate group. These compounds are prone to decarboxylation owing to the fact that this reaction can proceed via a six-membered pericyclic transition state.[14,15] In the structure with 3MG, we noticed that the ligand assumes a bent conformation, which is a prerequisite to attain such a geometry. Furthermore, the hydrophobic environment of the active site will decrease the acidity of the γ-carboxylate such that it is likely protonated, as is required for the mechanism shown in Figure 2e (bottom). Hence, we hypothesize that AibA/AibB catalyzes the decarboxylation of MG-CoA by exploiting the intrinsic instability of its substrate, forcing it to adopt the geometry and protonation state required for such a reaction. The resulting β,γ-unsaturated intermediate would then rearrange to DMA-CoA, either within the active site or after release from the enzyme.

To corroborate this hypothesis through structural methods, we investigated a number of CoA- or *N*-acetylcysteamine (SNAC) analogues of the postulated transition state (Figure S4), but co-crystallization with these compounds failed. However, we obtained a 1.6 Å resolution crystal structure of AibA/AibB in the presence of the transition-state mimic 2-methyl-1-cyclohexanecarboxylic acid (2m1c), a molecule that lacks the CoA moiety. In this structure, 2m1c adopts a chair conformation in which the carboxylate group occupies an axial position and binds the active site through interactions with the backbone amide atoms of $Val34^B$ and $Gly134^B$. The C2 methyl group is equatorial and points into a hydrophobic pocket formed by $Ala26^B$, $Leu89^B$, $Val101^B$, and $Leu138^B$. Interestingly, although a racemic mixture of *cis*- and *trans*-2m1c was used for soaking, only the (1*R*,2*S*)-*cis* isomer bound to the protein (Figure 2c and Figure S2C), thus indicating that the active site deforms the substrate in a specific way. This is also corroborated by comparing this structure with the 3MG complex. Both molecules bind AibA/AibB in a similar manner, which further supports the postulated intramolecular decarboxylation mechanism (Figure 2d).

In conclusion, our study reveals the mechanistic basis of an AibA/AibB-mediated pericyclic decarboxylation. To our knowledge, AibA/AibB is the first cofactor-free decarboxylase using such a mechanism. Furthermore, our results also show the importance of in-depth biochemical and structural analysis to identify and characterize novel enzymes that would otherwise be misannotated by sequence alignments and homology searches alone. In addition, the data shown here may be of interest for biotechnological applications because we established an effective procedure for the production of active AibA/AibB in *E. coli*. This process could be integrated into synthetic biology approaches to produce isobutene, a precursor for renewable biofuels and chemicals, from glucose by following Scheme S1 in the Supporting Information.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: biosynthesis · decarboxylation · enzymes · protein expression · structural biology

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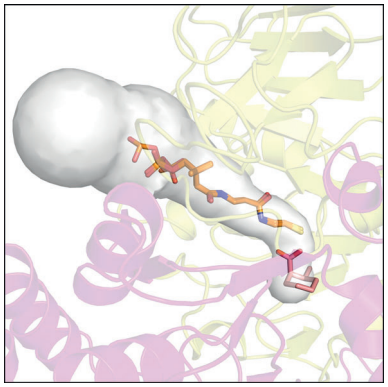

Based on structural and biochemical characterization of the unique cofactor-free decarboxylase AibA/AibB, an intramolecular decarboxylation mechanism supported by the geometry and hydrophobic character of the active site is proposed. This study opens up opportunities for synthetic biology approaches, since AibA/AibB is well suited to the bacterial production of isobutene, a precursor of renewable biofuels and chemicals.