Structural Basis of the Stereospecificity of Bacterial B_{12}-dependent 2-Hydroxyisobutyryl-CoA Mutase

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Background: Bacterial B_{12}-dependent 2-hydroxyisobutyryl-CoA mutase specifically catalyzes the isomerization of (S)-3-hydroxybutyryl- and 2-hydroxyisobutyryl-CoA.

Results: The crystal structure of 2-hydroxyisobutyryl-CoA mutase shows decisive differences in the active site when compared with the well studied methylmalonyl-CoA mutase.

Conclusion: Specificity toward (S)-3-hydroxybutyryl-CoA strongly depends on the active site amino acid Asp{sup A117}. Significance: This is the first structural characterization of a B_{12}-dependent mutase with α_{3}β_{2} organization isomerizing 2-hydroxyisobutyryl-CoA.

Bacterial coenzyme B_{12}-dependent 2-hydroxyisobutyryl-CoA mutase (HCM) is a radical enzyme catalyzing the stereospecific interconversion of (S)-3-hydroxybutyryl- and 2-hydroxyisobutyryl-CoA. It consists of two subunits, HcmA and HcmB. To characterize the determinants of substrate specificity, we have analyzed the crystal structure of HCM from Aquincola tertiari-carbonis in complex with coenzyme B_{12} and the substrates (S)-3-hydroxybutyryl- and 2-hydroxyisobutyryl-CoA in alternative binding. When compared with the well studied structure of bacterial and mitochondrial B_{12}-dependent methylmalonyl-CoA mutase (MCM), HCM has a highly conserved domain architecture. However, inspection of the substrate binding site identified amino acid residues not present in MCM, namely HcmA Ile{sup A90} and Asp{sup A117}. Asp{sup A117} determines the orientation of the hydroxyl group of the acyl-CoA esters by H-bond formation, thus determining stereospecificity of catalysis. Accordingly, HcmA D117A and D117V mutations resulted in significantly increased activity toward (R)-3-hydroxybutyryl-CoA. Besides interconversion of hydroxylated acyl-CoA esters, wild-type HCM as well as HcmA I90V and I90A mutant enzymes could also isomerize pivalyl- and isovaleryl-CoA, albeit at >10 times lower rates than the favorite substrate (S)-3-hydroxybutyryl-CoA. The nonconservative mutation HcmA D117V, however, resulted in an enzyme showing high activity toward pivalyl-CoA. Structural requirements for binding and isomerization of highly branched acyl-CoA substrates such as 2-hydroxyisobutyryl- and pivalyl-CoA, possessing tertiary and quaternary carbon atoms, respectively, are discussed.

Coenzyme B_{12}-dependent acyl-CoA mutases are a relatively small family of enzymes catalyzing carbon skeleton rearrangements through a unique radical mechanism (1). Thus far, methylmalonyl-CoA mutase (MCM), ethylmalonyl-CoA mutase (ECM), and isobutyryl-CoA mutase (ICM) have been characterized (2–4). In addition, a variant of ICM (IcmF) has been described as a fusion of ICM with the G-protein chaperon Meal (5), a paralog to the MCM-associated MeeB protein. Recently, we have characterized 2-hydroxyisobutyryl-CoA mutase (HCM) as the newest member of the acyl-CoA mutase family (6). All these radical enzymes catalyze reversible 1,2-rearrangements of the carboxylic acid skeleton of their CoA ester substrates (Fig. 1), e.g. the MCM-catalyzed interconversion of methylmalonyl- and succinyl-CoA, with very narrow substrate specificity. Mutases play a central role in primary metabolism, such as branched-chain amino acid catabolism in mammals (MCM) and the ethylmalonyl-CoA pathway for acetate assimilation in prokaryotes (ethylmalonyl-CoA mutase). In contrast, bacterial ICM seems to be mainly involved in synthesis of secondary metabolites, such as macrolide and polyether antibiotics (7, 8). However, a role of the ICM-like IcmF in bacterial catabolism of the prodrug component pivalic acid has recently been suggested (9). Likewise, HCM is employed in some bacteria for dissimilation of 2-hydroxysobutyric acid (6). This unusual short-chain carboxylic acid is formed during degradation of xenobiotic fuel oxygenates (10) but is also found in humans with lactic acidosis (11), and lysine 2-hydroxyisobutyrylation has recently been identified as a widely distributed histone mark (12). Besides these characterized enzymes, several not yet identified B_{12}-dependent mutases have been postulated to be involved in microbial degradation pathways for the anaerobic mineralization of alkanes and ethylbenzene (8), indicating that different subfamilies of acyl-CoA mutases may exist as key
enzymes in microbial degradation pathways of natural compounds as well as xenobiotics.

The unusual radical mechanism exchanging a hydrogen with a thioester group on vicinal carbon atoms (Fig. 1) has been thoroughly studied in MCM from the Gram-positive bacterium Propionibacterium freudenreichii subsp. shermanii (PfMCM) (13, 14). These studies showed that binding of the acyl-CoA substrate results in large conformational changes that cause the formation of an initial radical by homolysis of the adenosylcobalamin-cobalt bond in the coenzyme B12. This 5’-deoxyadenosyl radical abstracts a hydrogen atom from the substrate (Fig. 1). Then, the substrate radical undergoes 1,2-rearrangement by migration of the thioester group and re-abstracts the hydrogen from the adenosine to form the acyl-CoA product. A striking feature of all acyl-CoA mutases is that the coenzyme B12 and the CoA substrate are completely buried within the enzyme. The amino acids interacting with B12 and the CoA moiety of the substrate are highly conserved in all acyl-CoA mutases. In contrast, only very few residues seem to be characteristic for each mutase subfamily for specifically interacting with the acyl moiety of the substrate, namely Tyr89 and Arg207 in PfMCM (15, 16). These residues form several H-bonds with the free carboxyl group of the substrate and radical intermediate. In addition, the Tyr determines strict specificity toward the (R)-enantionter of methylmalonyl-CoA (15).

Based on sequence comparisons, residues homologous to Tyr89 and Arg207 of PfMCM have been identified as PheA80 and GlnA198 or any other residue as the free substrate carboxyl group does in MCM. Likewise, the preference for (S)-3-hydroxybutyryl-CoA found in HCM cannot be explained without a reliable structure model (6). In addition, differences in subunit organization (Fig. 2) might also lead to deviations from the thus far studied MCM structures. PfMCM and HsMCM are structurally closely related as coenzyme B12 and acyl-CoA binding sites are located on the same catalytically active subunit. PfMCM is heterodimeric, consisting of one catalytic subunit and a homologous but only regulatory subunit of similar size lacking essential binding domain residues (e.g., Tyr89 and Arg207 for acyl-CoA binding). HsMCM is homodimeric. In contrast to both PfMCM and HsMCM, however, in bacterial ICM and HCM as well as in recently described archaeal MCMs (19, 20), acyl-CoA and coenzyme B12 binding sites are distributed on two subunits of significantly different size (i.e., large and small subunits A and B, respectively).

Here, we present the crystal structure to 2.5 Å resolution of the complex between the two subunits of recombinant HCM (HcmA and HcmB), coenzyme B12, and the substrates (S)-3-hydroxybutyryl- and 2-hydroxyisobutyryl-CoA. In comparison with the structure of MCM, decisive differences in the active site could be identified, enabling the specific interconversion of (S)-3-hydroxybutyryl- and 2-hydroxyisobutyryl-CoA.

**EXPERIMENTAL PROCEDURES**

**Materials and Syntheses**—2-Hydroxyisobutyric acid (>98%) was purchased from Merck Schuchardt (Hohenbrunn, Germany). Anhydride of isovaleric acid (99%) was from ABCR GmbH & Co. KG (Karlsruhe, Germany). Anhydride of pivalic acid (≥98%), sodium salts of (R)-3-hydroxybutyric (96%) and (S)-3-hydroxybutyric (≥97%) acid, coenzyme B12 (≥97%), and CoA (≥93%) were purchased from Sigma-Aldrich (Steinheim, Germany). Isovaleryl- and pivalyl-CoA were prepared from their anhydrides (21). 2-Hydroxyisobutyryl-CoA, (R)-3-hydroxybutyryl-CoA, and (S)-3-hydroxybutyryl-CoA were synthesized from the free acid forms via thiophenyl esters (22).

**Cloning, Expression, and Purification of Recombinant Proteins**—Genes encoding for the large and small subunits of HCM from A. tertiaricarbonis L108 (hcmA and hcmB) were cloned into pASG-IBA43 vectors (IBA GmbH, Goettingen,
Germany) and chemically transformed into Escherichia coli TOP10. After growth in Luria-Bertani medium containing 100 mg liter$^{-1}$ ampicillin to an optical density at 550 nm of 0.5, induction was performed with 200 µg liter$^{-1}$ anhydrotetracycline for 3 h at 30 °C. Cells were harvested by centrifugation and suspended in 100 ml of wash buffer (100 mM Tris, 150 mM NaCl, pH 8.0) for further analysis. Likewise, hcmA mutant genes cloned into pASG-IBA43 were transformed into E. coli TOP10 and expressed as described above. Site-directed mutagenesis of the wild-type hcmA (GeneCust Europe) resulted in HcmA I90V, I90L, and I90A mutants (with the point mutations a268g, a268c, and a268g plus t269c, respectively) and in HcmA D117A I90V, I90L, and I90A mutants (with the point mutations a268g, a268c, and a268g plus t269c, respectively) and in HcmA D117A

For the purification of recombinant mutase subunits used for testing enzyme activities, crude extracts of induced E. coli cells were prepared by mechanical disruption using a mixer mill (MM 400, Retsch GmbH, Haan, Germany) with glass beads (212–300 µm, Sigma) at 30 s$^{-1}$ for 30 min. The recombinant HcmA and HcmB subunits were purified with the help of their His- and Strep-tags, respectively, as described previously (6). For protein purification of A. tertiaricarbonis HcmA and HcmB used for crystallization, the E. coli cells were resuspended in lysis buffer (20 mM NaH$_2$PO$_4$, 500 mM NaCl, 30 mM imidazole, 5% glycerol, pH 7.5), homogenized, and ultracentrifuged at 48,000 × g for 1 h. The supernatants were loaded onto a His-Trap (GE Healthcare) nickel affinity chromatography column and eluted with 250 mM imidazole. The pooled fractions were applied to a HiLoad 16/60 Superdex 200 prep grade (GE Healthcare) size-exclusion chromatography column using 20 mM Tris, 150 mM NaCl, 2 mM DTT, 5% glycerol, pH 7.5, as buffer and concentrated to 20 mg ml$^{-1}$.
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| Table 1 | Crystallographic data and refinement statistics |
|----------------------|-----------------------------------------------|
| **Parameter**        | **Value**                                    |
| X-ray source         | Bessy BL 14.1                                |
| Wavelength (Å)       | 0.918                                        |
| Temperature (K)      | 100                                          |
| Space group          | C2                                           |
| a/b/c (Å)            | 69.0/119.6/173.9                             |
| α/β/γ (%)            | 90.0/91.2/90.0                               |
| Resolution (Å)       | 24.63-2.5 (2.64-2.5)*                        |
| Rfree (%)            | 10.0 (48.9)                                  |
| Rwork (%)            | 14.2 (3.3)                                   |
| Completeness (%)     | 99.9 (100.0)                                 |
| No. of unique reflections | 48815 (7111)                              |
| Multiplicity         | 5.2 (5.2)                                    |
| Wilson B-factor (Å^2)| 27.2                                         |

**Refinement**

- Rfree/Rwork (%): 16.8/21.8
- r.m.s.d.: 0.010 Å
- Angles (%): 1.17

**Protein**

- Total: 49.2
- Chain A: 36.1
- Chain B: 58.7
- Chain C: 38.8
- Chain D: 77.4
- Water: 30.1

**Ligands**

- A800 (B12): 26.1
- B800 (B12): 47.7
- A801 (3HB): 34.0
- B801 (3HB): 56.2
- A802 (2HB): 32.2
- B802 (2HB): 56.7
- A803 (5AD; occ 0.5): 26.4
- Ramachandran plot
- Most favored (%): 92.7
- Allowed (%): 7.0
- Generously allowed (%): 0.3
- Disallowed (%): 0.0

**PDB ID**: 4R3U

- Values in brackets refer to the highest resolution shell.
- r.m.s.d.: root mean square deviation.
- B12, cobalamin; 3HB, (S)-3-hydroxybutyryl-CoA, 2HB, 2-hydroxyisobutyryl-CoA; 5AD, 5'-deoxyadenosyl residue.

### Results

**Heterotetrameric Assembly of HCM and Comparison with the MCM Structure**

The structure of HCM in complex with coenzyme B12 and the substrates (S)-3-hydroxybutyryl- and 2-hydroxyisobutyryl-CoA has been determined to 2.5 Å resolution (Fig. 3). In contrast to the structure of HsMCM and PfMCM in which two domains are connected by a long linker, HCM consists of two different subunits representing the MCM domains, HcmA for the N-terminal TIM barrel domain and HcmB for the C-terminal Rossmann fold domain (13–15, 18). Furthermore, HCM forms an αβγ-heterotetramer, in which the interface between the two protomers is formed entirely by HcmA (Fig. 3A). HCM and MCM share similar protein architectures with root mean square deviation values of 1.34/0.98 and 1.52/0.85 Å for the two HCM subunits to catalytically active subunits of PfMCM and HsMCM, respectively. In agreement, the active monomer from the heterodimeric PfMCM (Fig. 3B) and a monomer of the homodimeric HsMCM (Fig. 3C) fit well to the αβ-subunit assembly in HCM. In HCM, both αβ-protomers are functional (concerning the binding of cofactors and substrates in a catalytically competent arrangement), and the only difference between HCM and the MCM structures is the absence of the interdomain regions. A sequence alignment of HCM with PfMCM and HsMCM shows that they share sequence identities of 44 and 41% with HcmA and 43 and 42% with HcmB, respectively (see also sequence alignment of HCM with PfMCM and HsMCM including secondary structure elements in Fig. 4).

**Active Site Architecture**

The active site cavity of HcmA is formed by the β-strands of the TIM barrel domain and is closed by HcmB with the bound cobalt-corrin ring (Fig. 3A). For co-crystallization, (S)-3-hydroxybutyryl-CoA was added to the enzyme, but (S)-3-hydroxybutyryl-CoA as well as the product 2-hydroxyisobutyryl-CoA were included in the final model because the electron density indicates alternative binding of the two substrates in the active site in approximately equal occupancy (Fig. 5A). A similar situation of a mixture of both substrates (or substrate/product pair) bound to the active site was observed in the PfMCM structure (15). The carboxyl groups of both compounds form H-bonds to HisA245, which is homologous to His244 in MCM (Fig. 5B). In PfMCM, TyrA99 plays a central role in the reaction mechanism by forming an H-bond to the carboxyl groups of the ligands, and it has been suggested to be involved in the generation of the 5'-deoxyadenosyl radical by displacement of 5'-deoxyadenosine from the cobalt atom (14, 15). In HCM, TyrA99 is exchanged by IleA990, which is smaller than the tyrosine side chain and is not capable of forming H-bonds to the ligands. Instead, AspA117 replaces the free space and forms H-bonds with the hydroxyl group of both HCM substrates. AspA117 and IleA990 most likely displace the 5'-deoxyadenosyl from cobalt together with the bound substrate after HcmA/HcmB assembly. Similar to the situation in MCM, these residues would clash with the adenosyl group if the cofactor would be bound as in the open substrate-free enzyme form (14) (Fig. 5, B and C). The homologous residue in PfMCM is AlaA116, which is unable to form a similar interaction. In PfMCM, the carboxyl groups of methylmalonyl- and succinyl-CoA form salt bridges with ArgA207 (Fig. 5C) (15). In HCM, this residue is replaced by GlnA208, which is too short for H-bond interactions with (S)-3-hydroxybutyryl- or 2-hydroxyisobutyryl-CoA (Fig. 5C).

In the free coenzyme B12, the dimethylbenzimidazole is coordinated to the cobalt ion. As in the complex structures of chains within the asymmetric unit lie in the generously allowed region of the Ramachandran plot, but both residues exhibit well defined electron density.

**Mutase Assay**

Isomerization activity was assayed by directly measuring product formation employing an HPLC ion-pair chromatography system as described previously (6). Briefly, equimolar amounts (3 μM) of recombinant HcmA and HcmB were incubated in 50 mM potassium phosphate buffer (pH 6.6) amended with 833 μM coenzyme B12, 10 mM MgCl2, and 10% glycerol at 30 °C in the dark. After a 5-min preincubation, the reaction was started by adding acyl-CoA substrate. For stopping the reaction, samples were mixed with an equal volume of 100 mM acetate buffer (pH 3.5) and heated at 60 °C for 5 min prior to HPLC analysis. Kinetic parameters were calculated by nonlinear regression analysis applying the Michaelis-Menten equation (OriginPro 9).
PfMCM (13), this group is flipped into a deep pocket of HcmB and HisB18 is coordinated to the cobalt ion at a distance of 2.5 Å. This long coordinative bond is believed to weaken the cobalt-C bond of the other distal ligand (13), and it is stabilized by the protein environment; HisB18 makes H-bonding interactions with AspB16, whose charge is compensated by LysB12 as in MCM. The 5′-deoxyadenosyl ligand is completely displaced from the cobalt atom, and it is bound with an occupancy of 0.5.

Structural Basis for Substrate Stereospecificity of HCM—As described above, AspA117 of HCM fixes the conformation of the substrates 2-hydroxyisobutyryl- and (S)-3-hydroxybutyryl-CoA by forming H-bonds to their hydroxyl groups. Via this interaction, AspA117 is probably also important for the stereospecificity favoring the interconversion of 2-hydroxyisobutyryl- and the (S)-enantiomer of 3-hydroxybutyryl-CoA. In the observed substrate binding mode, the hydrogen atoms that need to be abstracted for catalytic turnover are well positioned for abstraction. The hydrogens are at 2.3–2.5 Å distance to the C5′ of 5′-deoxyadenosyl, which bears the unpaired electron after dissociation from the cobalt ion (Fig. 5, B and C). In the R-isomer of 3-hydroxybutyryl-CoA, the methyl group would change position with the hydrogen atom if the hydroxyl group maintains the favorable interaction with AspA117, and the hydrogen would not be positioned for abstraction.

Isomerization Activity of HCM Variants—Residues IleA90 and AspA117 are likely the most important amino acids for substrate specificity of HCM when compared with MCM. Mutating IleA90 to Tyr or Phe results in a complete loss of HCM activity (6). The HCM crystal structure suggests that these larger side chains would clash with the catalytically competent conformation of AspA117 (Fig. 5C). Surprisingly, also the substitution of IleA90 against the similar amino acid Val dramatically decreased the reaction rate and substrate affinity (6). In line with this, the mutations HcmA I90L and I90A resulted in a significant reduction in conversion rates and 6- to >300-fold diminution of the catalytic efficiencies (Table 2) for the main substrates of the wild-type enzyme, (S)-3-hydroxybutyryl- and 2-hydroxyisobutyryl-CoA. Interestingly, reduction of activity was smallest for the isomerization of 2-hydroxyisobutyryl-CoA with the substitution of the IleA90 by the relatively small Ala, retaining about 50% of the wild-type $V_{\text{max}}$. In addition, with (R)-3-hydroxybutyryl-CoA, this mutant showed a 6-fold higher catalytic efficiency than the wild-type enzyme, although the $V_{\text{max}}$ of 2.6 nmol min$^{-1}$ mg$^{-1}$ was still quite low. As predicted

![Structure of 2-Hydroxyisobutyryl-CoA Mutase](image-url)
from the active site architecture, the nonconservative mutation HcmA D117V resulted in reversion of stereospecificity, now clearly favoring \((R)-3\)-hydroxybutyryl-CoA at a \(V_{\text{max}}\) of 125 nmol min\(^{-1}\) mg\(^{-1}\) that is close to the wild-type rate previously determined for the \((S)\)-enantiomer (Table 2). With the latter substrate, about 3-fold reduced activities were observed, indicating that a catalytically competent orientation is more uncommon but still possible, i.e. positioning of the hydroxyl group of the \(S\)-isofrom in the neighborhood to Val\(^{117}\). The HcmA D117A enzyme, on the other hand, showed similar \(V_{\text{max}}\) values of about 20–30 nmol min\(^{-1}\) mg\(^{-1}\) for all three hydroxacyl-CoA substrates. However, due to the quite low \(K_m\) value obtained for \((R)-3\)-hydroxybutyryl-CoA, the catalytic efficiency exceeded 200 mmol min\(^{-1}\) min\(^{-1}\), which is the highest value among all HCM variants and substrates tested (Tables 2 and 3).

As pivalyl-CoA is another potential substrate for coenzyme \(B_{12}\)-dependent mutases possessing a similar branching complexity as the HCM substrate 2-hydroxyisobutyryl-CoA, interconversion of this acyl-CoA and isovaleryl-CoA was tested (Table 3). The wild-type HCM could isomerize these substrates at 6–8% of the \(V_{\text{max}}\) value of 140 nmol min\(^{-1}\) mg\(^{-1}\) previously obtained for its most favorite substrate \((S)-3\)-hydroxybutyryl-CoA. In addition, the HcmA 190V and 190A mutant enzymes also isomerized pivalyl- and isovaleryl-CoA at rates close to the wild-type \(V_{\text{max}}\) values. Due to lower \(K_m\) values, the HcmA 190A mutant even possesses slightly higher catalytic efficiencies than the wild-type enzyme. The HcmA 190L mutant, however, did not show any significant activities with pivalyl- and isovaleryl-CoA. In contrast, conversion rates of pivalyl-CoA by the HcmA D117V enzyme were much higher than observed with the wild-type and HcmA 190 mutant enzymes, showing a \(V_{\text{max}}\) value of about 100 nmol min\(^{-1}\) mg\(^{-1}\).

**DISCUSSION**

In HCM, the coenzyme \(B_{12}\)- and substrate-binding domains form two separate protein chains in contrast to \(p\)MCM and HsMCM. However, the missing linkage between the two domains does not influence the protein assembly. The substrate-bound structure of HCM reveals significant differences in substrate binding when compared with MCM.

**Determinants of HCM Substrate Specificity**—The most significant structural difference between MCM, ICM, and HCM substrates is the type of the substituents attached to the thioester-linked carbon atom (Fig. 1). Aside from the thioester carbonyl carbon, the linked carbon atom forms covalent bonds with methyl and carboxyl carbons in methylmalonyl-CoA, but the fourth substituent is a hydrogen atom. A similar situation is found with isobutyryl-CoA where only the carboxyl group of MCM substrates is replaced by a methyl residue. In contrast, only in 2-hydroxyisobutyryl-CoA the thioester-linked carbon is attached to four non-hydrogen substituents. The structure of HCM reveals that the orientation of the hydroxyl group of HCM substrates corresponds to the hydrogen attached to the thioester-linked carbon of MCM substrates (Fig. 5C). A consequence of this bulkier structure of the HCM substrates is that the active site amino acid Tyr\(^{89}\) of MCM is replaced by the smaller Ile\(^{90}\) in HCM, which is in close neighborhood to the hydroxyl group of the substrate. In line with this, MCM is absolutely specific to \((R)\)-methylmalonyl-CoA, as the methyl group of \((S)\)-methylmalonyl-CoA would have a similar orientation as the hydroxyl residue in the HCM substrates and would clash with the aromatic ring of Tyr\(^{89}\) (15). The demand of additional space for the acyl residue in HCM substrates could explain the complete loss of activity previously observed with HcmA 190Y and 190F mutant forms (6), in addition to the displacement of Asp\(^{117}\), which is important for substrate binding. Surprisingly, even the conservative mutation HcmA 190V caused a dramatic decrease in HCM activity (6). Now, we have demonstrated that HcmA 190A and 190L mutations also resulted in significant reduction in catalytic efficiency for the HCM main substrates 2-hydroxyisobutyryl- and \((S)-3\)-hydroxybutyryl-CoA (Table 2). Possible explanations are steric clashes of the introduced Leu\(^{90}\) side chain with neighboring HcmA residues resulting in a repositioning of some protein residues important for the catalytic cycle and substrate binding and, on the other hand, fewer stabilizing interactions in the case of the smaller side chains of Val\(^{90}\) and Ala\(^{90}\). The larger side chain of Ile\(^{90}\) is involved in van der Waals interactions with the bound substrates, and its absence may cause more flexibility within the acyl group of the CoA ester substrates.

In \(p\)MCM, H-bond formation of the substrate carboxyl group with Tyr\(^{89}\) and Arg\(^{207}\) plays a central role in substrate binding, radical formation, and intermediate stabilization (15, 16). By analogy, a similar role can be expected from the hydroxyl group of 2-hydroxyisobutyryl- and \(3\)-hydroxybutyryl-CoA. In fact, Asp\(^{117}\) has been identified in the HCM structure as the important active site residue specifically interacting with the acyl moiety by forming an H-bond with the hydroxyl group of HCM substrates (Fig. 5B). Obviously, this interaction also determines the stereospecificity of HCM. Only in the \((S)\)-enantiomer the hydrogen atom is positioned properly toward the C5‘ of \(5\)‘-deoxyadenosine for its abstraction. If the \((R)\)-enantiomer is modeled in a catalytically competent binding mode with the hydrogen atom facing the C5‘ of \(5\)‘-deoxyadenosine, the methyl group would form close contacts with Asp\(^{117}\). If the hydroxyl group of the \((R)\)-enantiomer is positioned in a favorable interaction with Asp\(^{117}\), the H-atom is not properly positioned for hydrogen abstraction. Thus, a catalytically competent low energy binding mode is only available for the \((S)\)-enantiomer. Consequently, the \(R\)-isomer is converted with a nearly 1000-fold lower catalytic efficiency than \((S)-3\)-hydroxybutyryl-CoA (6). With the mutant HcmA 190A, catalytic efficiency toward the \((R)\)-enantiomer is slightly improved, likely due to increased freedom for the Asp\(^{117}\) conformation to avoid unfavorable close contacts to the methyl group of the substrate. Residue Gln\(^{208}\) does not seem to play as essential a role in substrate binding as the homologous residue Arg\(^{207}\) in
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A

B

C
TABLE 2

Kinetic parameters $V_{\text{max}}$, $K_m$, and $k_{\text{cat}}$ and deduced catalytic efficiency $k_{\text{cat}}/K_m$ of reconstituted HCM subunits HcmA (wild-type Ile$^{90}$, Asp$^{117}$) and mutants as indicated and wild-type HcmB incubated with 2-hydroxyisobutyryl-CoA and both 3-hydroxybutyryl-CoA enantiomers at pH 6.6 and 30 °C

| Variant and substrate | $V_{\text{max}}$ (nmol min$^{-1}$ mg$^{-1}$) | $K_m$ (μM) | $k_{\text{cat}}$ (min$^{-1}$) | $k_{\text{cat}}/K_m$ (ms$^{-1}$/μM) |
|-----------------------|------------------------------------------|-----------|----------------|----------------------------------|
| Wild-type HcmA$^a$ (Ile$^{90}$, Asp$^{117}$) | 140 ± 5.6 | 128 ± 22 | 12 ± 0.5 | 90 ± 16 |
| Wild-type HcmB | 2.4 ± 0.15 | 1.660 ± 367 | 0.20 ± 0.01 | 0.12 ± 0.03 |
| HcmA I90V$^b$ | 24 ± 1.1 | 1,760 ± 240 | 2.0 ± 0.1 | 1.1 ± 0.16 |
| HcmA I90V | 2.48 ± 0.02 | 663 ± 12 | 0.21 ± 0.002 | 0.31 ± 0.01 |
| HcmA I90A | 1.5 ± 0.1 | 433 ± 56 | 0.12 ± 0.01 | 0.28 ± 0.04 |
| HcmA D117V | 125 ± 4.8 | 67.0 ± 10.2 | 10.4 ± 0.4 | 155 ± 24 |
| HcmA D117V | 18.4 ± 0.6 | 14.6 ± 3.8 | 1.53 ± 0.05 | 105 ± 27 |
| HcmA D117V | 32.0 ± 1.5 | 42.0 ± 8.1 | 2.66 ± 0.12 | 63.2 ± 12.5 |
| HcmA D117V | 19.7 ± 0.5 | 7.5 ± 1.7 | 1.64 ± 0.04 | 220 ± 49 |

$^a$ Previously determined values (6).
$^b$ Activities obtained for (R)-3-hydroxybutyryl-CoA with HcmA I90V and I90L mutants reconstituted with wild-type HcmB were below detection limit (<0.01 nmol min$^{-1}$ mg$^{-1}$).

TABLE 3

Kinetic parameters $V_{\text{max}}$, $K_m$, and $k_{\text{cat}}$ and deduced catalytic efficiency $k_{\text{cat}}/K_m$ of reconstituted HCM subunits HcmA (wild-type Ile$^{90}$, Asp$^{117}$) and mutants as indicated and wild-type HcmB incubated with pivalyl- and isovaleryl-CoA at pH 6.6 and 30 °C

| Variant and substrate | $V_{\text{max}}$ (nmol min$^{-1}$ mg$^{-1}$) | $K_m$ (μM) | $k_{\text{cat}}$ (min$^{-1}$) | $k_{\text{cat}}/K_m$ (ms$^{-1}$/μM) |
|-----------------------|------------------------------------------|-----------|----------------|----------------------------------|
| Wild-type HcmA (Ile$^{90}$, Asp$^{117}$) | 11.8 ± 0.73 | 374 ± 46 | 0.98 ± 0.06 | 2.6 ± 0.4 |
| Pivalyl-CoA | 8.20 ± 0.34 | 288 ± 18 | 0.68 ± 0.03 | 2.4 ± 0.2 |
| Isovaleryl-CoA | 12.3 ± 0.96 | 977 ± 122 | 1.02 ± 0.08 | 1.0 ± 0.2 |
| HcmA I90V | 6.74 ± 0.54 | 329 ± 69 | 0.56 ± 0.05 | 1.7 ± 0.4 |
| Pivalyl-CoA | 9.4 ± 0.1 | 235 ± 4 | 0.79 ± 0.01 | 3.4 ± 0.1 |
| Isovaleryl-CoA | 6.5 ± 0.2 | 147 ± 14 | 0.54 ± 0.02 | 3.7 ± 0.4 |
| HcmA D117V | 101 ± 3.9 | 43.5 ± 6.3 | 8.83 ± 0.32 | 192 ± 29 |

$^a$ Activities obtained for pivalyl- and isovaleryl-CoA with the HcmA I90L mutant reconstituted with wild-type HcmB were below detection limit (<0.01 nmol min$^{-1}$ mg$^{-1}$). Conversion by the HcmA D117A mutant and conversion of isovaleryl-CoA by the HcmA D117V mutant have not been tested in this study.

$^a$ Previously determined values (6).
$^b$ Activities obtained for (R)-3-hydroxybutyryl-CoA with HcmA I90V and I90L mutants reconstituted with wild-type HcmB were below detection limit (<0.01 nmol min$^{-1}$ mg$^{-1}$).

PIMCM. The MCM substrates (R)-methylmalonyl- and succinyl-CoA are not turned over by HCM as Arg$^{207}$ and Tyr$^{89}$ are not available for salt bridge and H-bonding interactions with the carboxyl groups (Fig. 5C). The homologous side chain of Gln$^{208}$ in HCM is probably too short to replace Arg$^{207}$ of MCM at least for a direct H-bonding interaction. Consequently, HCM does not catalyze rearrangement of these substrates (6). As expected, the mutation HcmA D117V resulted in reversion of stereospecificity likely due to a now favored orientation of the methyl group of (R)-3-hydroxybutyryl-CoA to Val$^{117}$ when compared with a similar positioning of the hydroxyl group of the S-isomer. Nevertheless, the high conversion rates of the mutant enzyme obtained for the R-isomer are somewhat surprising. Considering the orientation of (R)-3-hydroxybutyryl-CoA in the HcmA D117V mutant, Gln$^{208}$ would be the only candidate for H-bond interaction, and it might interact with the hydroxyl group via a water molecule. In addition, hydrophobic interactions with Ile$^{90}$ and Val$^{117}$ support substrate binding. In summary, active site amino acids Ile$^{90}$ and Asp$^{117}$ determine substrate specificity and catalytic efficiency in HCM. Consequently, both residues are conserved in all HCMs identified thus far, whereas mutases of the other subfamilies possess different amino acids at these positions (Fig. 6).

Reflections on Pivalyl-CoA Mutase (PCM) and ICM Activity—For the bacterial dissimilation of pivalic acid, another coenzyme B$_{12}$-dependent mutase has been postulated isomerizing pivalyl-CoA into isovaleryl-CoA (32). Like 2-hydroxyisobutyryl-CoA, pivalyl-CoA possesses four non-hydrogen atoms attached to the thiester-linked carbon. However, in contrast to the former acyl-CoA ester, both R residues depicted in Fig. 1 are methyl groups in the case of pivalyl-CoA. In the HCM structure, placing pivalyl-CoA analogous to 2-hydroxy-

FIGURE 5. Active site architecture of the bacterial HCM from A. tertiaria carbonis. A, stereo figure of the omit electron density map (blue, 3.0σ$_{\text{rms}}$, where r.m.s. indicates root mean square) for the coenzyme B$_{12}$ (light pink) and the substrates 2-hydroxyisobutyryl- (dark brown) and (S)-3-hydroxybutyryl-CoA (orange). The adenosyl radical (light pink), which is part of the coenzyme B$_{12}$ complex, is depicted in dim colors because it is only partially occupied (occupancy factor 0.5). B, stereoscopic view of the interaction of coenzyme B$_{12}$ and the substrates 2-hydroxyisobutyryl- (dark brown) and (S)-3-hydroxybutyryl-CoA (orange) with HCM. The substrates are shown in different color intensities in each figure part (left or right stereo part). When viewed in stereo, alternating eye-switching results in an optimal impression of the binding modes of the two substrates. The adenosyl radical is depicted in dim colors as in panel A. Hydrogen atoms best positioned for abstraction by the adenosyl radical are colored white. Residues of HCM that interact with the acyl groups of the substrates or coenzyme B$_{12}$ are colored in green or red, respectively. C, stereoscopic view of the superposition of HCM (green carbon atoms) in complex with 2-hydroxyisobutyryl-CoA (brown) with PIMCM with bound (R)-methylmalonyl-CoA (light blue). For a better view, the substrates are shown in different color intensities as in panel B. Interacting PIMCM residues are colored in gray.

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isobutyryl-CoA would result in unfavorable close interactions between the substrate methyl group and the carboxyl group of Asp\textsuperscript{117}. In line with this, isomerization activity with pivalyl- and isovaleryl-CoA turned out to be low for HCM, reaching only less than 10% of the (S)-3-hydroxybutyryl-CoA conversion rate. However, this observed PCM activity of HCM is quite high when compared with the isovaleryl-CoA isomerization rates. Accordingly, the HcmA D117V mutation resulted in a 10-fold increased PCM activity of HCM.

Analysis of the structure of HCM and comparison with the architecture previously found in the MCM subfamily (14, 18) has shed new light on active site architecture and determinants of substrate specificity in this fascinating group of radical enzymes. In the future, tailor-made mutases might be developed for the rearrangement of carboxylic acids relevant for industrial and pharmaceutical production, in particular for the generation or identification of enzymes with efficient HCM, PCM, and ICM activity (7, 33–35). Detailed structural information of the substrate interaction helps in the rational enzyme design of these industrially relevant biocatalysts.

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