Engineered and Native Coenzyme B_{12}-dependent Isovaleryl-CoA/Pivalyl-CoA Mutase*  

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Background: IcmF exhibits low isovaleryl-CoA/pivalyl-CoA mutase (PCM) activity.  
Results: IcmF mutants designed to enhance PCM activity were susceptible to inactivation prompting a bioinformatics search for a "bona fide" PCM.  
Conclusion: A B_{12}-dependent PCM was identified, cloned, and expressed and exhibited PCM activity.  
Significance: The newly discovered PCM could be useful in bioremediation and biosynthetic reactions.

Adenosylcobalamin-dependent isomerases catalyze carbon skeleton rearrangements using radical chemistry. We have recently demonstrated that an isobutyryl-CoA mutase variant, IcmF, a member of this enzyme family that catalyzes the interconversion of isobutyryl-CoA and n-butyl-CoA also catalyzes the interconversion between isovaleryl-CoA and pivalyl-CoA, albeit with low efficiency and high susceptibility to inactivation. Given the biotechnological potential of the isovaleryl-CoA/pivalyl-CoA mutase (PCM) reaction, we initially attempted to engineer IcmF to be a more proficient PCM by targeting two active site residues predicted based on sequence alignments and crystal structures, to be key to substrate selectivity. Of the eight mutants tested, the F598A mutation was the most robust, resulting in an ~17-fold increase in the catalytic efficiency of the PCM activity and a concomitant ~240-fold decrease in the isobutyryl-CoA mutase activity compared with wild-type IcmF. Hence, mutation of a single residue in IcmF tuned substrate specificity yielding an ~4000-fold increase in the specificity for an unnatural substrate. However, the F598A mutant was even more susceptible to inactivation than wild-type IcmF. To circumvent this limitation, we used bioinformatics analysis to identify an authentic PCM in genomic databases. Cloning and expression of the putative AdoCbl-dependent PCM with an αβ2 heterotetrameric organization similar to that of isobutyryl-CoA mutase and a recently characterized archaean methylmalonyl-CoA mutase, allowed demonstration of its robust PCM activity. To simplify kinetic analysis and handling, a variant PCM-F was generated in which the αβ subunits were fused into a single polypeptide via a short 11-amino acid linker. The fusion protein, PCM-F, retained high PCM activity and like PCM, was resistant to inactivation. Neither PCM nor PCM-F displayed detectable isobutyryl-CoA mutase activity, demonstrating that PCM represents a novel 5’-deoxyadenosylcobalamin-dependent acyl-CoA mutase. The newly discovered PCM and the derivative PCM-F, have potential applications in bioremediation of pivalic acid found in sludge, in stereospecific synthesis of C5 carboxylic acids and alcohols, and in the production of potential commodity and specialty chemicals.

5’-Deoxyadenosylcobalamin (AdoCbl),3 a derivative of cobalamin, is used as a cofactor by enzymes that catalyze 1,2 rearrangement reactions using radical chemistry (1–5). The distinguishing feature of the cofactor is its cobalt-carbon bond, which has a bond dissociation energy of ~31 kcal/mol (6) and holds the key to its utility as a transient source of radicals for initiating chemically challenging isomerization reactions (7). Within the family of AdoCbl-dependent isomerases, the acyl-CoA mutases are the most rapidly growing subclass and several new members have been described in recent years (Fig. 1A) (3). Of these, methylmalonyl-CoA mutase (MCM), which catalyzes the interconversion of methylmalonyl-CoA to succinyl-CoA, is the best studied (1, 5, 8). MCM is the most widely distributed of the AdoCbl-dependent isomerases and is found in organisms ranging from bacteria to man. Relatives of MCM, which catalyze carbon skeleton rearrangements on structurally similar substrates, include isobutyryl-CoA mutase (ICM) (9), ethylmalonyl-CoA mutase (ECM) (10), and 2-hydroxyisobutyryl-CoA mutase (HCM) (11) (Fig. 1A).

Our laboratory has described a variant of ICM, IcmF (for isobutyryl-CoA mutase fused), which is encoded by many bacterial genomes (12). In IcmF, the separate B_{12r} and substrate-binding subunits are fused in a single polypeptide chain together with a G-protein chaperone (Fig. 1B). Thus, IcmF is composed of the three domains, an N-terminal AdoCbl-binding domain, a middle G-protein domain, and a C-terminal substrate-binding domain and displays both

3 The abbreviations used are: AdoCbl, 5’-deoxyadenosylcobalamin; ECM, ethylmalonyl-CoA mutase; HCM, 2-hydroxyisobutyryl-CoA mutase; ICM, isobutyryl-CoA mutase; IcmF, isobutyryl-CoA mutase fused with its G-protein chaperone; MCM, methylmalonyl-CoA mutase; OH_{2}Cbl, aquocobalamin; PCM, pivalyl-CoA mutase; PCM-F, engineered fusion construct of PCM; LIC, ligation-independent cloning; TEV, tobacco etch virus; MBP, maltose-binding protein.
variants. ICM and PCM are heterotetramers in which two small B12-binding subunits interact with two large substrate-binding subunits. IcmF is a natural variant in which the large and small subunits have been fused via an 11-amino acid linker as described under "Experimental Procedures." In this study, we engineered CmlcmF to enhance its acyl-CoA mutase activity at the expense of its isobutyryl-CoA mutase activity by introducing mutations at Phe-598 and Gln-742 (Fig. 2B). The F598A mutation switched the substrate specificity of IcmF and enhanced the catalytic efficiency of the isovaleryl-CoA mutase over the native isobutyryl-CoA mutase activity ~4000-fold. However, the mutation also enhanced the propensity of the enzyme to inactivation during turnover. This prompted us to use bioinformatics analysis to identify a PCM from Xanthobacter autotrophicus and herein, we report its initial characterization.

Experimental Procedures

Materials—AdoCbl, GTP, GDP, isobutyryl-CoA, isovaleryl-CoA, and DL-3-hydroxybutyryl-CoA were purchased from Sigma. Valeric acid was purchased from Fluka. Dithiothreitol and tris(2-carboxyethyl)phosphine hydrochloride were from Gold Biotechnology (St. Louis, MO). All other chemicals were purchased from Sigma or Fisher Scientific, and were used without further purification.

Site-directed Mutagenesis of CmlcmF—Mutagenesis was performed using the QuickChange XL site-directed mutagenesis kit (Agilent Technologies) using the CmlcmF cloned in the pMCSG7 plasmid as a template. The following set of forward primers in which the mutagenic codon is underlined were syn-
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thesized by Integrated DNA Technologies (Coralville, IA) and used to introduce missense mutations: F598A, 5'-CCC ACG CGC ATG GCC GCG GCC GAG GG-3'; F598G, 5'-CCC ACG CGC ATG GCC GCG GCC GAG GG-3'; F598I, 5'-CCC ACG CGC ATG ATC GGC GGC GAG-G3'; F598L, 5'-CCC ACG CGC ATG TTA GGC GGC GAG GAT G-3'; F598Y, 5'-CCC ACG CGC ATG TTA GGC GGC GAG GAT G-3'.

The sequences of the reverse primers were complementary to those of the forward primers. The mutations were confirmed by nucleotide sequence determination at the University of Michigan DNA Sequencing Core.

Expression and Purification of CmIcmF—Recombinant wild-type and mutant IcmFs were expressed and purified as described previously (13).

Cloning of PCM Subunits—The genomic DNA from X. autotrophicus strain Py2 (ATCC BAA-1158) was purchased from ATCC (Manassas, VA). The large and small subunits of PCM were PCR-amplified using Phusion High-Fidelity DNA polymerase (Agilent Technologies) using the following primers containing NdeI and HindIII restriction sites (underlined): large subunit (forward): 5'-AGT ATA CAT ATG AAC CAG GCC GCC GTG-3', and large subunit (reverse), 5'-TGT TCA TGC ATG TTA TTA TCA TGG GAT G-3'; small subunit (forward), 5'-CTA GTA CAT ATG ATC CAT GCC GGC ACG-3', and small subunit (reverse), 5'-A GAT AAG CGT TCA TGG GTT TGC CTC CG-3'.

Subsequently, the resulting vector was used as a template for ligation-independent cloning (LIC) for the large subunit with the following primers, 5'-TAC TTC CAA TTC AAT GCA ATG AAC CAG GCC GCC GTG-3' (forward) and 5'-T TAT CCA CTT CCA ATG TTA TTA TCA GCC GGC GTG-3' (reverse), and subcloned into a LIC vector, which introduces a His6-MBP tag and tobacco etch virus (TEV) protease cleavage site at the N terminus of the expressed protein. Three additional residues, Ser-Asn-Ala, were added to the N terminus of the expressed protein following removal of the His6-SUMO tag.

Expression and Purification of PCM and PCM-F—Escherichia coli BL21(DE3) was transformed with the vector expressing the large subunit of PCM and grown overnight at 37 °C in 5 ml of Luria-Bertani medium containing ampicillin (100 μg/ml). Then, 6 liters of the same medium containing ampicillin was inoculated with the starter culture and grown at 37 °C. After 4 h, when the A600 had reached 0.6–0.8, the temperature was reduced to 15 °C. The cultures were induced with 0.1 mM isopropyl β-D-1-thiogalactopyranoside, and the cells were harvested 20 h later. The cell pellets were stored at −80 °C until use. The cell pellets were suspended in 150 ml of buffer containing 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 20 mM imidazole, 0.2 mg/ml of lysozyme, 1 mM PMSF and one protease inhibitor mixture tablet (Roche Applied Science). The cell suspension was stirred at 4 °C for 40 min and then sonicated (power setting = 6) on ice for 10 min at 30-s intervals separated by 60-s cooling periods. The sonicate was centrifuged at 35,000 × g for 30 min, and the supernatant was loaded onto a Ni-Sepharose 6 Fast Flow column (2.5 × 8 cm, GE Healthcare) pre-equilibrated with Buffer A (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 20 mM imidazole). The column was washed with 300 ml of Buffer A containing 20 mM imidazole and eluted with 300 ml of a linear gradient ranging from 20 to 300 mM imidazole in Buffer A. The fractions of interest were identified by SDS-PAGE analysis, pooled, and concentrated to 5 ml, and dialyzed overnight against 1 liter of dialysis buffer containing 100 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 1 mM 2-mercaptoethanol and His6-MBP tag was cleaved with TEV protease (1 mg of TEV protease/100 mg of protein). The dialyzed protein was loaded onto an amylose resin column (New England Biolabs) to remove His6-MBP tag, and the flow-through fraction was collected and loaded on to a Superdex 200 column (120 ml, GE Healthcare) pre-equilibrated with 50 mM HEPES-NaOH, pH 7.5, 100 mM NaCl. The fractions of interest were pooled, concentrated, frozen in liquid nitrogen, and stored at −80 °C until further use. The His6-tagged small subunit of PCM was expressed as described for the large subunit with the exception that kanamycin (50 μg/ml) was used as the antibiotic and purified using a Ni-Sepharose 6 Fast Flow column and followed by gel filtration using a Superdex 200 column. PCM-F was expressed as a fusion with a His6-tagged SUMO protein and expressed and purified using the same procedures as described above for the large subunit with the exception of the amylose resin column, which was excluded.

Enzyme Assays—Enzyme activity was measured using 50 mM HEPES-NaOH, pH 7.5, containing 100 mM NaCl and 10 mM MgCl2 in a total volume of 0.6 ml. The reaction mixture contained 0.5–20 μM enzyme, 100 μM AdoCbl, and 0.1–5 mM

Construction of an Expression Plasmid for Fusion Protein between Large and Small PCM Subunits—To generate a fusion of the large and small subunits of PCM (PCM-F), a co-expression system was initially constructed using pETDuet-1 vector (Novagen), in which the large subunit was inserted into cloning site 1 (MCS1) using XbaI and HindIII restriction sites, and the small subunit was inserted into MCS2 using NdeI and XhoI restriction sites. Subsequently, the second translation initiation site between MCS1 and MCS2 was deleted and an 11-mer containing 0.5–20 μM enzyme, 100 μM AdoCbl, and 0.1–5 mM
Isovaleryl-CoA or isobutyryl-CoA ± 1 mM nucleotides (GTP or GDP) at 37 °C. The enzymes were preincubated with AdoCbl (± 1 mM nucleotides in the case of IcmF), and the reaction was started by addition of substrate. At various time points (0.5–60 min), 100-μl aliquots were removed and quenched with 50 μl of 2 N KOH containing 0.2 mM valeric acid used as an internal standard. Following addition of 50 μl of H2SO4 (15%, v/v), the reaction mixture was saturated with solid NaCl and extracted with ethyl acetate (125 μl). The extract was analyzed directly by gas chromatography (GC, Agilent Technologies) using a 0.25-μm DB-FFAP (30-m × 0.25 mm inner diameter) capillary column (Agilent Technologies). A 5-μl sample was injected in the pulsed splitless mode. The oven temperature was initially at 80 °C. Following the sample injection, the temperature was raised to 150 °C at a rate of 10 °C/min and maintained at 150 °C for 2 min. Retention times for the compounds of interest were as follows: isobutyric acid, 5.85 min; pivalic acid, 5.99 min; n-butric acid, 6.5 min; isovaleric acid, 6.96 min; and valeric acid, 7.78 min.

To determine the kinetic parameters for the isovaleryl-CoA mutase and isobutyryl-CoA mutase activities of IcmF, the initial rate of the reaction was plotted against substrate concentration and fitted to the Michaelis-Menten equation, where
\[ v = \frac{V_{\text{max}} \times [S]}{K_m + [S]} \]

To determine the kinetic parameters of isovaleryl-CoA mutase activity of PCM, the initial rate of the reaction was plotted against substrate concentration and fitted to the Hill equation, where
\[ v = \frac{V_{\text{max}} \times [S]^n}{K_m + [S]^n} \]

Enzyme-monitored Turnover—Spectral changes in AdoCbl bound to IcmF and PCM were monitored by UV-visible spectroscopy at 25 °C in 50 mM HEPES-NaOH, pH 7.5, containing 100 mM NaCl and 10 mM MgCl2. Substrates (1 mM final concentration of isovaleryl-CoA or isobutyryl-CoA) were added to 50 μM AdoCbl-loaded enzyme. With IcmF, the influence of the G-protein domain on catalytic turnover was checked by addition of 5 mM GTP or GDP to the reaction mixture. The amount of cob(II)alamin formed under steady-state turnover conditions was calculated from the decrease in absorbance at 525 nm upon substrate addition using a value of Δε525 nm of -4.8 mm⁻¹ cm⁻¹ (20). During the course of the reaction, AdoCbl was gradually converted to enzyme-bound aquocobalamin (OH₂Cbl) as indicated by the appearance of the 351-nm absorption peak. The increase in absorption at 351 nm (A) was fitted to a single exponential function, \( A = A_0 + A_1 (1 - e^{-kt}) \) where k is the observed rate constant for inactivation, A0 is the initial absorbance at 351 nm, t is time in minutes, and A₁ is the amplitude.

Isothermal Titration Calorimetry—Isothermal titration calorimetry experiments were performed in triplicate as described previously (12) and the data were analyzed using Microcal ORIGIN software. Briefly, binding of AdoCbl to PCM was monitored as follows. Enzyme (10 μM small subunit of PCM or PCM-F) in 50 mM HEPES-NaOH buffer, pH 7.5, containing 100 mM NaCl was titrated with 30 × 10-μl aliquots of a 0.4 mM solution of AdoCbl at 20.0 °C. The calorimetric signals were integrated and the data were well fitted to a single-site binding model to estimate the equilibrium association constant, \( K_a \), and the binding enthalpy, \( \Delta H^\circ \). The Gibbs free energy of binding, \( \Delta G^\circ \), and the entropic contribution to the binding free energy, \(-T\Delta S^\circ\), were calculated using Equations 1 and 2.

\[ \Delta G^\circ = -RT \ln K_a \]  
\[ \Delta G^\circ = \Delta H^\circ - T \Delta S^\circ \]

Bioinformatics Analysis—The integrated microbial genomes database web-based tools were used for BLAST search and operon analysis (21). A multiple sequence alignments were constructed using a stand-alone version of ClustalX version 2.1.

Results

Structure-based Redesign of IcmF—Our efforts at enhancing the catalytic activity of IcmF with isovaleryl-CoA as substrate were guided by sequence comparisons of IcmF with other acyl-CoA mutases (10, 11) and by the crystal structures of IcmF (17) and MCM (16, 22). Sequence comparisons revealed the presence of a putative acyl-CoA mutase annotated as MCM-like in which Leu and Asn substitute Phe-598 and Gln-742, respectively, in IcmF (Fig. 2A). Hence, these two residues were targeted by multiple missense mutations (Table 1). To better accommodate the longer isovaleryl-CoA substrate, we introduced residues with smaller side chains at Phe-598 (Ala, Gly, Ile, Leu, and Val) and Gln-742 (Ala, Leu, and Asn). The yield of each of the purified mutants (~5.0 mg/liter of culture) was similar to that of wild-type IcmF and the purity of each mutant protein was judged to be >95% by SDS-PAGE analysis (data not shown). The mutants, like wild-type IcmF, eluted as a single peak by gel filtration chromatography with a molecular mass of 279 kDa, consistent with being homodimers.

Isovaleryl-CoA Mutase and Isobutyryl-CoA Mutase Activities of IcmF—The isovaleryl-CoA mutase and isobutyryl-CoA mutase activities of the mutants were compared with wild-type IcmF (Table 1). The F598A mutation simultaneously enhanced isovaleryl-CoA mutase activity 6-fold (0.18 ± 0.02 μmol min⁻¹ mg⁻¹) and diminished isobutyryl-CoA activity >600-fold (0.039 ± 0.003 μmol min⁻¹ mg⁻¹), with respect to wild-type IcmF (0.030 ± 0.001 μmol min⁻¹ mg⁻¹ and 24 ± 3 μmol min⁻¹ mg⁻¹, respectively). Substitution of Phe-598 with the nonaromatic hydrophobic residues isoleucine, leucine, and valine, either maintained or increased isovaleryl-CoA mutase activity, whereas isobutyryl-CoA mutase activity was diminished albeit with less dramatic effects than the F598A mutation. In addition, mutations at Phe-598 decreased the \( K_m \) for isovaleryl-CoA and therefore increased the catalytic efficiency (\( k_{cat}/K_m \)) of the isovaleryl-CoA mutase reaction compared with wild-type IcmF. The activity of the F598G mutant was not detectable with either substrate. Mutation of Gln-742 to alanine, leucine, or asparagine, resulted in undetectable isovaleryl-CoA mutase activity and significantly diminished isobutyryl-CoA mutase activity (Table 1).

Inactivation Kinetics of IcmF Mutants—The active sites in the CmlcmF dimer exhibit identical affinity for AdoCbl (\( K_D = 0.27 ± 0.01 \mu M, n = 2.1 ± 0.1 \)) in contrast to the different affinities reported for the Geobacillus kaustophilus IcmF (\( K_{D1} = 0.081 ± 0.014 \mu M \) and \( K_{D2} = 1.98 ± 0.42 \mu M \)) (12). To assess whether the low or undetectable activity of several of the CmlcmF mutants in Table 1 was due to their enhanced propen-
**TABLE 1**

Comparison of the kinetic parameters for the isovaleryl-CoA mutase and isobutyryl-CoA mutase activities of wild-type and mutant IcmFs, PCM, and PCM-F

Enzyme activities were determined at 37°C and represent the average of at least three independent experiments ± S.D. The activity of PCM was measured in the presence of a 3:1 ratio of small:large subunit. Wild-type and the mutants in the first column refer to IcmF.

| Enzyme | k_{cat} \( \text{min}^{-1} \) | K_{m} \( \mu M \) | k_{cat}/K_{m} | k_{cat} \( \text{min}^{-1} \) | K_{m} \( \mu M \) | k_{cat}/K_{m} |
|--------|-------------------------------|-----------------|---------------|-------------------------------|-----------------|---------------|
| Wild-type | 3.7 ± 0.1                       | 1.4 ± 0.1       | 2.6           | 2900 ± 400                             | 1.1 ± 0.3                   | 2600           |
| F598A   | 22 ± 2                             | 0.51 ± 0.15     | 43            | 4.8 ± 0.4                              | 0.45 ± 0.11               | 11             |
| F598G   | ND                                   | ND              | ND            | ND                                        | ND                         | ND             |
| F598I   | 3.6 ± 0.2                         | 0.58 ± 0.12     | 6.2           | 8.4 ± 0.3                              | 0.36 ± 0.05              | 23             |
| F598L   | 8.4 ± 0.8                         | 0.56 ± 0.16     | 15            | 76 ± 4                          | 0.89 ± 0.10              | 85             |
| F598V   | 15 ± 1                              | 0.62 ± 0.16     | 24            | 12 ± 2                          | 0.67 ± 0.23            | 18             |
| Q742A   | ND                                   | ND              | ND            | 18 ± 2                          | 0.79 ± 0.23            | 23             |
| Q742L   | ND                                   | ND              | ND            | ND                                        | ND                         | ND             |
| Q742N   | ND                                   | ND              | ND            | ND                                        | ND                         | ND             |
| PCM     | 14 ± 3                              | 0.37 ± 0.13     | 38            | 110 ± 10                            | 2.4 ± 0.3                 | 46             |
| PCM-F   | 23 ± 1                              | 0.20 ± 0.01     | 115           | ND                                        | ND                         | ND             |

*The k_{cat} values for IcmF, PCM, and PCM-F were calculated per α, αβ, and α unit, respectively of each enzyme.

ND is not detected. The detection limit of the GC assay is 5 pmol of product.
sity for inactivation, the spectrum of AdoCbl bound to IcmF was monitored following addition of isovaleryl-CoA (Fig. 3). For these experiments, IcmF was reconstituted with 2 eq of AdoCbl and the absence of free AdoCbl was confirmed by analyzing the spectrum of the filtrate obtained after concentrating the sample mixture using an Amicon concentrator (50-kDa cutoff).

Addition of isovaleryl-CoA to wild-type holo-IcmF resulted in formation of the intermediate, cob(II)alamin, as evidenced by the decrease in absorbance at 530 nm and increase at 471 nm (Fig. 3A). Using a \( \Delta \varepsilon_{525} \) of \(-4.8 \text{ mm}^{-1} \text{ cm}^{-1}\) (20), cob(II)alamin was estimated to represent \~20\% of the cofactor under steady-state turnover conditions (Table 2). In contrast, AdoCbl was quantitatively converted to cob(II)alamin when isovaleryl-CoA was added to F598A (Fig. 3B) or to the F598I/L or Q742A mutants (Table 2). Accumulation of all the cofactor as cob(II)alamin indicates a change in the reaction coordinate such that the barrier to one or more steps following cob(II)alamin formation and preceding reforma-

![Figure 3](https://example.com/fig3.png)

**TABLE 2**

Comparison of inactivation kinetics for wild-type and mutant IcmFs, PCM, and PCM-F

Inactivation rates for OH\(_2\)Cbl formation were determined in the presence of isovaleryl-CoA at 25°C and represent the average of at least three independent experiments ± S.D.

| Enzyme          | \( k_{\text{obs}} \) (min\(^{-1}\)) | Cob(II)alamin*/% |
|-----------------|-------------------------------------|------------------|
| Wild-type IcmF  | 0.035 ± 0.001                       | 20               |
| F598A IcmF      | 0.12 ± 0.02                         | 100              |
| F598G IcmF      | ND†                                 | ND               |
| F598I IcmF      | 0.080 ± 0.007                       | 100              |
| F598L IcmF      | 0.14 ± 0.007                        | 100              |
| F598V IcmF      | 0.077 ± 0.007                       | 90               |
| Q742A IcmF      | 0.10 ± 0.01                         | 100              |
| Q742L IcmF      | 0.0090 ± 0.0002                     | <10              |
| Q742N IcmF      | ND‡                                 | ND               |
| PCM*            | 0.014 ± 0.002                       | 5                |
| PCM-F           | 0.016 ± 0.002                       | 5                |

* The concentration of the catalytic intermediate, cob(II)alamin was calculated 2–5 min after adding isovaleryl-CoA under aerobic conditions. Errors were <10%.

† ND, not determined due to rapid formation of OH\(_2\)Cbl following addition of isovaleryl-CoA.

‡ The inactivation rate of PCM was measured in the presence of a 1:2 or 1:3 ratio of small:large subunit and were found to be indistinguishable.

Because cob(II)alamin can be oxidized to OH\(_2\)Cbl, its increased accumulation in the mutants could lead to an increased propensity for OH\(_2\)Cbl formation. OH\(_2\)Cbl is a hallmark of inactivation in AdoCbl-dependent enzymes (23) and is characterized by an increase in absorption at 351 and 530 nm (Fig. 3C). The F598A mutant inactivates 3-fold more rapidly than wild-type IcmF as monitored by the increase in absorbance at 351 nm (Fig. 3D, Table 2).

The F598G and Q742N mutants showed rapid conversion of AdoCbl to OH\(_2\)Cbl under aerobic conditions without detectable accumulation of the cob(II)alamin intermediate (Table 2). Hence, enzyme-monitored turnover indicates that the IcmF mutants engineered to have enhanced isovaleryl-CoA mutase activity are simultaneously more susceptible to inactivation. The only exception is Q742L IcmF, which showed a lower proportion of the cofactor in the cob(II)alamin state under steady-
state turnover conditions and a lower rate of \( \text{OH}_2\text{Cbl} \) formation (Table 2). However, Q742L IcmF did not exhibit isovaleryl-CoA mutase activity (Table 1).

**Effects of Nucleotides and Reductants on the Isovaleryl-CoA Mutase Activity of IcmF**—Next, the ability of nucleotides that bind to the G-protein domain of IcmF, on isovaleryl-CoA mutase activity was assessed with the most active mutant, F598A. The G-protein domain interacts with both the \( B_12 \) and substrate-binding domains in IcmF (17). The presence of GTP or GDP increased the maximal isovaleryl-CoA mutase activity of wild-type IcmF by \( \times 4 \)-fold (Fig. 4A). However, the same conditions had virtually no effect on the isovaleryl-CoA mutase activity of the F598A mutant (Fig. 4B). Furthermore, whereas the nucleotides stabilized wild-type IcmF against inactivation, they had little or no effect on the F598A mutant (Fig. 4, C and D).

In a further attempt to stabilize IcmF against inactivation, the effect of several reductants was tested. Both DTT and 2-mercaptoethanol enhanced the maximal isovaleryl-CoA mutase activity of wild-type IcmF \( \sim 5 \)-fold and F598A IcmF \( \sim 2 \)-fold, respectively, whereas GSH and tris(2-carboxyethyl)phosphine hydrochloride were without effect (data not shown).

**Identification of a Putative Pivalyl-CoA/Isovaleryl-CoA Mutase**—Given the high susceptibility of the most active IcmF mutant to inactivation, we used bioinformatics analysis to identify a “bona fide” isovaleryl-CoA/pivalyl-CoA mutase. We examined sequences that are annotated in the databases as MCM-like proteins but differ in the two active site residues that are predicted to be important for substrate specificity especially in organisms with multiple copies of acyl-CoA mutases (24, 25). Specifically, we identified a group of genes that encode a substrate-binding subunit where Leu and Asn substitute Phe-598 and Gln-742, respectively, in IcmF. We concentrated on \( X. \) autotrophicus strain Py2 where \( X_{\text{aut}}_5043 \) and \( X_{\text{aut}}_5044 \) encode the large and small subunits, respectively, of an AdoCbl-dependent mutase, tentatively identified as a PCM. In the large subunit of the putative PCM, Leu-87 and Asn-204 correspond to Phe-598 and Gln-742 in IcmF, respectively (Fig. 2). In addition, a gene encoding a putative G-protein chaperone (\( X_{\text{aut}}_5042 \)) is present in the same operon. The bacterial MeaB and human CblA are G-protein chaperones that gate docking of AdoCbl to bacterial and human MCM, respectively, whereas the corresponding G-protein in IcmF is fused between the small and large subunits (Fig. 1B). The putative PCM is similar in organization to ICM, which exists as an \( \alpha_2\beta_2 \) heterotetramer with the small and large subunits binding AdoCbl and substrate, respectively (9).

**Purification and Kinetic Properties of PCM**—The recombinant large subunit of PCM was purified as a His\(_6\)-MBP-tagged protein. Following cleavage of the tag, the large subunit was obtained with a yield of \( \sim 4 \) mg of protein/liter of culture. The band observed by SDS-PAGE analysis corresponds to the predicted mass of 62.3 kDa for the large subunit (Fig. 5A). Size exclusion chromatography on a calibrated Superdex 200 column yielded an estimated molecular mass of 135 kDa, consistent with the large subunit being a homodimer (not shown).
FIGURE 5. Characterization of PCM and PCM-F. A, the purity of the large and small subunits of PCM and PCM-F were judged by SDS-PAGE analysis. B, dependence of isovaleryl-CoA mutase activity of PCM on the molar ratio of the small:large subunit. The reaction mixture contained 1 mM isovaleryl-CoA in 50 mM HEPES-NaOH buffer, pH 7.5, 100 mM NaCl, 10 mM MgCl₂ at 37 °C and varying ratios of the small/large subunit as described under “Experimental Procedures.” C, dependence of isovaleryl-CoA mutase activity of PCM (open circles) and PCM-F (closed circles) on the concentration of isovaleryl-CoA (0.1–1.0 mM) in 50 mM HEPES-NaOH buffer, pH 7.5, 100 mM NaCl, 10 mM MgCl₂ at 37 °C. The ratio of the small to large subunit of PCM was fixed at 3:1. The kinetic parameters derived from this analysis are reported in Table 1. D, time dependence of the inactivation of PCM (closed circles) and PCM-F (closed triangles). The inactivation of wild-type and F598A IcmF (Fig. 3D) are also included for comparison.

The recombinant small subunit of PCM was purified as an N-terminal His<sub>6</sub>-tagged protein and ~6 mg of highly pure protein were obtained per liter of culture (Fig. 5A). The small subunit eluted with an apparent mass of 20 kDa by gel filtration chromatography, suggesting that it exists as a monomer based on the predicted mass of the polypeptide of 17.7 kDa. Gel filtration of a 1:1 mixture of the large and small subunits of PCM in the presence of AdoCbl, showed no evidence of complex formation, indicating weak interaction between the subunits under these conditions.

Next, the dependence of the isovaleryl-CoA mutase activity of PCM was assessed at increasing small:large subunit ratios (Fig. 5B). Maximal activity was observed at an ~1:1 ratio. The specific activity (0.18 ± 0.04 μmol min⁻¹ mg⁻¹ of protein) and steady-state kinetic parameters for PCM were determined at a 3:1 molar ratio of the small:large subunit. The activity of PCM showed a sigmoidal dependence on the concentration of isovaleryl-CoA (Fig. 5C). From a Hill plot analysis of the data, the following values were estimated: K<sub>m</sub> for isovaleryl-CoA = 0.37 ± 0.13 mM, k<sub>cat</sub> = 14 ± 3 min⁻¹, and n (Hill coefficient) = 1.4 ± 0.4, indicating positive cooperativity. Unlike IcmF, the activity of PCM was resistant to inactivation (Table 2 and Fig. 5D).

Purification and Kinetic Characteristics of PCM-F—Because the weak interaction between the large and small subunits of PCM complicates kinetic analysis, we created a variant in which the large and small subunits were linked in a single polypeptide by an 11-amino acid long (Gly-Gln)<sub>5</sub>-Gly sequence to give the fusion protein, PCM-F (Fig. 1B). This strategy has been used previously to study an AdoCbl-dependent isomerase, glutamate mutase, which like PCM, exhibits an α<sub>2</sub>β<sub>2</sub> stoichiometry (26). Highly pure PCM-F was obtained at a yield of ~2 mg of protein/liter of culture (Fig. 5A). Gel filtration chromatography yielded an apparent mass of 168 kDa indicating that PCM-F with a subunit molecular mass of 78.8 kDa, exists as an α<sub>2</sub>β<sub>2</sub> homodimer.

The activity of PCM-F showed a sigmoidal dependence on the concentration of isovaleryl-CoA (Fig. 5C). From a Hill plot analysis of the data, the following values were estimated: K<sub>m</sub> for isovaleryl-CoA = 0.20 ± 0.01 mM, k<sub>cat</sub> = 23 ± 1 min⁻¹, and n (Hill coefficient) = 2.0 ± 0.3. The catalytic efficiency (k<sub>cat</sub>/K<sub>m</sub>) of PCM-F is 3-fold higher than for PCM.

Binding of AdoCbl to PCM and PCM-F—The energetics of AdoCbl binding to the PCM and PCM-F were monitored by isothermal titration calorimetry (Table 3). Binding of AdoCbl to the small subunit of PCM (K<sub>d</sub> = 12.5 ± 1.9 μM) exhibits a ΔG of −6.6 ± 0.1 kcal/mol and is enthalpically favored. The K<sub>d</sub> for AdoCbl is lower for PCM-F (4.9 ± 0.3 μM) than for PCM and exhibits a ΔG of −7.3 ± 0.2 kcal/mol, which is also enthalpically driven. Hence, the presence of the “built-in” large subunit in PCM-F enhances the affinity for AdoCbl ~2.6-fold.

Discussion

Our understanding of pivalic acid metabolism in nature is very limited. Some bacteria are known to mineralize pivalic acid to carbon dioxide (14). Some bacteria use pivalic acid as a
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TABLE 3 Thermodynamic parameters for the binding of AdoCbl to PCM

| n       | $K_d$  | $\Delta H$ | $\Delta S$ | $\Delta G$ |
|---------|--------|------------|------------|------------|
|         | $\mu$  | kcal/mol   | kcal/mol   | kcal/mol   |
| PCM-small subunit | 0.9 ± 0.1 | 12.5 ± 1.9 | -14.4 ± 2.3 | -7.8 ± 2.4 | -6.6 ± 0.1 |
| PCM-F   | 0.9 ± 0.1 | 4.9 ± 0.3  | -18.2 ± 10.1 | -11.1 ± 10.1 | -7.3 ± 0.2 |

The experiments were performed in 50 mM HEPES-NaOH, pH 7.5, 100 mM NaCl at 20°C as described under “Experimental Procedures.” The data represent the mean ± S.D. of three independent experiments.

Starter unit and incorporate it into tertiary-butyl fatty acids, iso-even or anteiso-fatty acids and in Streptomyces avermitilis, into the antibiotic avermectin (27). In addition, pivalic acid (or pivalyl-CoA) might be formed during catabolism of marine sponge-derived polythynamides in which the tert-butyl modification of threonine is introduced post-translationally (28). Isovaleryl-CoA is produced during leucine catabolism via the branched chain keto acid dehydrogenase or in myxobacteria, via a mevalonate-dependent isoprenoid biosynthesis pathway involving 3-hydroxy-3-methylglutaryl-CoA synthase (29). In several bacteria, the gene encoding IcmF is coregulated with those involved in branched-chain amino acid degradation (30) or fatty acid catabolism (13). The potential for utilizing a catalyst with isovaleryl-CoA/pivalyl-CoA isomerase activity for metabolic engineering to produce branched C4 and C5 building blocks or the corresponding alcohol derivatives, is untapped.

Until this study, IcmF was the only known albeit inefficient, catalyst with isovaleryl-CoA/pivalyl-CoA isomerase activity, and with high susceptibility to inactivation (13). The PCM described in this study is derived from X. autotrophicus strain Py2, a facultative aerobe that was first isolated from black pool sludge, has versatile metabolic capabilities, and is able to utilize 2-hydroxyisobutyric acid, propene, and trichloroethylene, 1-butene among others, as carbon sources (31). The large subunit of PCM has Leu and Asn residues that are distinct from all previously characterized ones, are important for substrate binding, are highlighted in dots (33). Although the ligase is presumably important for converting the acid to a CoA ester needed for the AdoCbl-dependent isomerase activity, the role of PCM in X. autotrophicus remains to be established. In E. coli, 14 genes are involved in the phenylactic acid degradation pathway (32).

Most acyl-CoA mutases exhibit fairly stringent substrate specificities, which appear to be dictated by a limited number of differences in key active site residues. In an effort to exploit the measurable but low isovaleryl/pivalyl-CoA mutase activity reported for wild-type IcmF (13), we rationally targeted two residues, which appear to be dictated by a limited number of differences in key active site residues. Of the individual missense mutations, F598A IcmF was the most active and led to an ~4000-fold enhancement of the isovaleryl-CoA mutase relative to the isobutyril-CoA mutase activity compared with wild-type enzyme (Table 1). However, F598A IcmF and the other mutants were even more susceptible than wild-type IcmF to inactivation during turnover (Table 2), a problem that was only marginally improved by the presence of reductants, DTT, and 2-mercaptoethanol.

A similar switch in substrate selectivity has been previously reported for MCM where the corresponding active site residues, Tyr-89 and Arg-207, were targeted by mutagenesis (33). The double mutant, Y89F/R207Q, mimicked the corresponding active site residues in ICM (and the then unknown IcmF). In contrast to wild-type MCM, the double mutant bound the ICM substrates, $n$-butyryl-CoA and isobutyryl-CoA, but instead of catalyzing their isomerization, promoted inactivation via a suicidal internal electron transfer mechanism. Among the mutants characterized in this study, the F598I IcmF resembles HCM with Ile-598 and Gln-742 corresponding to Ile-90 and Gln-208 in HCM (11). Therefore, we examined the possibility that F598I IcmF exhibits HCM activity. However, DL-3-hydroxybutyryl-CoA was not converted to product as judged by an HPLC-based assay (data not shown) although cob(II)alamin formation was seen indicating that the first step, cobalt-carbon bond homolysis, had occurred. These results indicate that residues that dictate the narrow substrate specificity of acyl-CoA mutases are distinct from those that promote the 1,2 rearrangement reactions. Alternatively, mutations that permit accom-

FIGURE 6. PCM sequence comparison and gene organization. A, multiple sequence alignment of substrate-binding domains of PCMs. PCMs from X. autotrophicus (Xaut_5043), Burkholderia phenoliruptrix (focus tag BPACDRAFT_03564), Cycloclastics sp. (focus tag Ga0055576_00391), and Nocardia spp. sp. (focus tag CF8_0950) are shown. The two conserved residues (Leu-87 and Asn-204, Xaut_5043). B, organization of the PCM-encoding operon from X. autotrophicus. The genes involved in the operon are annotated as paaK (phenylacetate-CoA ligase-like, Xaut_5040), paaD (phenylactic acid degradation protein-like, Xaut_5041), a mevalolog encoding a G-protein chaperone (Xaut_5042), pcmA (small subunit of PCM, Xaut_5043), pcmB (small subunit of PCM, Xaut_5044), and tetR (a transcriptional regulator, Xaut_5045).
modation of non-native substrates also lead to a misalignment in the active site residues so that unwanted side reactions such as oxidation of cob(II)alamin, are suppressed. Loss of reaction fidelity is seen in mutants in other AdoCbl-dependent enzymes, e.g. diol dehydratase, which catalyzes the conversion of 1,2-propanediol and 1,2-ethanediol to the corresponding aldehydes, and undergoes mechanism-based inactivation in the presence of glycerol. Interestingly, the S301A and Q336A mutations in the catalytic subunit of diol dehydratase confer resistance to glycerol-dependent inactivation relative to the wild-type enzyme indicating that these residues are involved in inactivation in the native enzyme (34).

The susceptibility of AdoCbl-dependent radical enzymes to irreversible inactivation during catalysis necessitates their reliance on chaperones for their reactivation. In the subclass of AdoCbl-dependent enzymes to which diol dehydratase belongs, reactivating factors mediate an ATP-dependent exchange of enzyme-bound OH2Cbl with free AdoCbl (23, 35). These reactivases have sequence similarity to DnaK and other members of the Hsp70 family of molecular chaperones and lower sequence similarity to the large subunits of corresponding mutases (23). In contrast, the G-protein chaperones associated with AdoCbl-dependent acyl-CoA mutases belong to the G3E family of P-loop metallochaperones (36). In MCM where the role of the G-protein chaperone MeA8 is best characterized (37), the chaperone uses GTP hydrolysis to power expulsion of cob(II)alamin when 5’-deoxyadenosine is lost from the active site (37). MeA8 diminishes the inactivation rate of the mutase ~15- and ~3-fold in the presence of GTP and GDP, respectively (20). The human ortholog of MeA8 is CbIAl and it is reported to prevent inactivation and increase the enzymatic activity of human MCM (38, 39). In IcmF, which has a built-in G-protein chaperone, GTP and GDP protect against inactivation in wild-type but not in F598A IcmF (Fig. 4, Table 2) indicating that this residue might be important for communication between the mutase and G-protein domains. The GTPase activity of IcmF is not affected in the Phe-598 mutants (kcat is 30 – 40 min−1), which is comparable with that of wild-type IcmF (34 ± 2 min−1 at 37 °C).

In summary, our efforts to rationally redesign the IcmF active site to improve its isovaleryl-CoA mutase activity yielded a protein that was a better catalyst but also more susceptible to inactivation. They also led to characterization of the first AdoCbl-dependent PCM exhibiting isovaleryl-CoA mutase activity, which was resistant to inactivation. We engineered a variant in which the two weakly interacting subunits of PCM were fused into a single polypeptide to obtain PCM-F, which was also highly active as an isovaleryl-CoA mutase. PCM-F could be useful in applications ranging from bioremediation to stereospecific synthesis of C5 carboxylic acids and alcohols.

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