Novel Coenzyme B_{12}-dependent Interconversion of Isovaleryl-CoA and Pivalyl-CoA*

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5'-Deoxyadenosylcobalamin (AdoCbl)-dependent isomerases catalyze carbon skeleton rearrangements using radical chemistry. We have recently characterized a fusion protein that comprises the two subunits of the AdoCbl-dependent isobutyryl-CoA mutase flanking a G-protein chaperone and named it isobutyryl-CoA mutase fused (IcmF). IcmF catalyzes the interconversion of isobutyryl-CoA and n-butyryl-CoA, whereas GTPase activity is associated with its G-protein domain. In this study, we report a novel activity associated with IcmF, i.e. the interconversion of isovaleryl-CoA and pivalyl-CoA. Kinetic characterization of IcmF yielded the following values: a $K_m$ for isovaleryl-CoA of 62 ± 8 μM and $V_{max}$ of 0.021 ± 0.004 μmol min$^{-1}$ at 37 °C. Biochemical experiments show that an IcmF in which the base specificity loop motif NKXD is modified to NKXE catalyzes the hydrolysis of both GTP and ATP. IcmF is susceptible to rapid inactivation during turnover, and GTP conferred modest protection during utilization of isovaleryl-CoA as substrate. Interestingly, there was no protection from inactivation when either isobutyryl-CoA or n-butyryl-CoA was used as substrate. Detailed kinetic analysis indicated that inactivation is associated with loss of the 5'-deoxyadenosine moiety from the active site, precluding reformation of AdoCbl at the end of the turnover cycle. Under aerobic conditions, oxidation of the cob(II)alamin radical in the inactive enzyme results in accumulation of aquacobalamin. Because pivalic acid found in sludge can be used as a carbon source by some bacteria and isovaleryl-CoA is an intermediate in leucine catabolism where isovaleric acid is an intermediate.

**Background:** IcmF is a fusion between a coenzyme B_{12}-dependent isobutyryl-CoA/n-butyryl-CoA isomerase and a G-protein chaperone.

**Results:** IcmF also isomerizes isovaleryl-CoA to pivalyl-CoA and is partially protected from inactivation in the presence of GTP.

**Conclusion:** The isovaleryl-CoA mutase activity of IcmF might be important in leucine catabolism where isovaleric acid is an intermediate.

**Significance:** IcmF might be critical for microbial bioremediation of the anthropogenic compound pivalic acid.

**Methy1malonyl-CoA mutase (MCM)** and isobutyryl-CoA mutase (ICM) are two closely related 5'-deoxyadenosylcobalamins (AdoCbl)-dependent isomerases that catalyze 1,2 rearrangements of methylmalonyl-CoA to succinyl-CoA and isobutyryl-CoA to n-butyryl-CoA, respectively (Fig. 1) (1, 2). MCMs are widely distributed in nature, ranging from bacteria and Archaea to animals, including humans. ICMs were initially believed to be restricted to the genus *Streptomyces*, which belongs to the Actinobacteria phylum (1, 2). With our discovery of IcmF, the fusion protein between ICM and its G-protein chaperone, the known distribution of ICM has expanded to include four more phyla: Proteobacteria, Bacteroidetes, Firmicutes, and Spirochaetes (3). The only known function of ICM in the genus *Streptomyces* is its participation in polyketide biosynthesis (4). In contrast, the relatively wide distribution of IcmF in diverse organisms points to a broader range of roles in bacterial metabolism that remain to be elucidated.

A family of enzymes that are similar in their primary sequence to MCM catalyzes AdoCbl-dependent carbon skeleton rearrangements and include, in addition to ICM, 2-hydroxyisobutyryl-CoA mutase (HCM) (5) and ethylmalonyl-CoA mutase (ECM) (6) (Fig. 1). The three-dimensional structure of these proteins is expected to resemble the overall structure of MCM (7, 8). The B_{12}-binding domain of MCM exhibits a typical Rossman-like fold, and the AdoCbl cofactor is bound in a “base-off/His-on” conformation (9). The substrate-binding domain is a triose-phosphate isomerase barrel comprising a core of eight αβ-repeats (8). A limited number of key amino acid substitutions in the active site distinguish these closely related enzymes and accommodate chemical differences in the substrate (6, 10). Previous studies from our laboratory had pointed out that the two key substitutions that accommodate the switch from a carboxylate to a methyl group in the substrates of MCM versus ICM are Tyr → Phe and Arg → Gln, respectively (Figs. 2 and 3) (3). The Phe and Arg substitutions are conserved in all ICM/IcmFs (Figs. 2 and 3). In contrast, in HCM, the corresponding amino acids that interact with substrate are Ile and Gln, respectively. Thus, although the Gln res-
idue is conserved in both ICM/IcmF and HCM, the Phe is substituted by Ile to accommodate the bulkier 2-hydroxyisobutyryl-CoA substrate (Figs. 2 and 3).

In contrast to ICM, ECM, which catalyzes the interconversion of ethylmalonyl-CoA and methylsuccinyl-CoA, is very similar to MCM (6). It has been proposed that two key substitutions in the active site of ECM dictate specificity for the bulkier ethylmalonyl-CoA substrate: a His and an Asn residue in MCM are replaced by Gly and Pro, respectively (6) (Fig. 3). Notably, the Tyr and Arg residues in the active site of MCM that interact with the carboxylate moiety of the substrate are also conserved in ECM (Fig. 3).

In B12-dependent isomerases, AdoCbl serves as a radical reservoir, and a common first step that initiates the isomerization reactions is homolytic cleavage of the cobalt-carbon bond (Fig. 4), leading to formation of the 5′-deoxyadenosyl radical and a paramagnetic cob(II)alamin species (1, 11). Inadvertent side reactions of the reactive radical intermediates render AdoCbl-dependent enzymes susceptible to inactivation (12). Alternatively, inactivation can result from escape of the 5′-deoxyadenosine intermediate during the catalytic cycle (13). In both cases, inactivation results from the inability to reform AdoCbl from the 5′-deoxyadenosyl and cob(II)alamin radicals at the end of the turnover cycle. MeaB, the G-protein chaperone of MCM, protects against inactivation in the presence of GTP (13, 14). A similar role for the homologous G-protein chaperone of ICM, MeaI, has not been studied. In IcmF, the MeaI domain is sandwiched between the AdoCbl- and substrate-binding domains (3). In this study, we report a novel AdoCbl-dependent 1,2 rearrangement reaction catalyzed by IcmF and demonstrate that in the presence of GTP the isovaleryl-CoA mutase activity of IcmF is partially protected from the inactivation.

EXPERIMENTAL PROCEDURES

Materials

AdoCbl, ADP, AMPPNP, ATP, GDP, GTP, isobutyryl-CoA, n-butyryl-CoA, isovaleryl-CoA, dl-3-hydroxybutyryl-CoA, and pivalic acid were purchased from Sigma. Tris(2-carboxyethyl)phosphine hydrochloride was from GoldBio (St. Louis, MO). Valeryl-CoA was purchased from Crystal Chem (Downers Grove, IL). A pET28a vector expressing IcmF from *Cupriavidus metallidurans* was a generous gift from the laboratory of Dr. Catherine Drennan (Massachusetts Institute of Technology).

DNA Manipulations

Cloning of IcmF—In a previous study, we used IcmF from *Geobacillus kaustophilus* cloned into pET30 Ek/LIC expression vector (3). The S-tag, which is located just downstream of the N-terminal His tag in pET30 Ek/LIC, was removed by subcloning the full-length IcmF DNA into the ligation-independent cloning vector pMCSG7 (15). The insert was amplified with the following primers: forward, 5′-TACTTCCAATCCAATGCCATGGCGCACATTTACCGTCC-3′; and reverse, 5′-TTATCCTATCTAAGCCTGACGGCCTTTTCCAGGCTTTAG-3′. The sequence of the reverse primer was complementary to the sequence of the forward primer. All con-
**IcmF Is a Pivalyl-CoA Mutase**

**ATPase/GTPase Assays**

NTPase activity of IcmF was measured by HPLC as described previously (3) or by a modification of the malachite green assay involving inclusion of citrate in the reaction mixture (16). Briefly, the color reagent was prepared by mixing 4.2% ammonium molybdate in 4 n HCl with 0.045% malachite green hydrochloride (1:3, v/v). The resulting solution was shaken for 30 min at 250 rpm in a Falcon tube and filtered through a 0.2-μm Milipore filter. After that, 10% Tween 20 solution (v/v) was added (200 μl for every 100 ml of color reagent). This solution was stable for a week at 4°C with only a minor increase in the background absorbance at 650 nm. The reaction was performed in Buffer A in a total volume of 0.6–1 ml containing 0.5–2.5 μM IcmF or 10–20 μM K213A IcmF, 10 mM MgCl₂, and nucleotides (0.02–1.2 mM GTP or 0.02–6 mM ATP). At the desired time points, 200-μl aliquots were removed, and the reaction was quenched with 20 μl of 2 n trichloroacetic acid (TCA). After centrifugation, 150 μl of supernatant was added to 750 μl of 2 M trichloroacetic acid (TCA). The resulting solution was dried in an oven (to remove traces of moisture) for 4 h at 120°C.

**IcmF Assay**

A GC-based assay was used to measure both ICM activity and the new, isovaleryl-CoA/pivalyl-CoA mutase activity (3). In all assays, apoenzyme was preincubated with AdoCbl ± nucleotides, and the reaction was started by addition of substrate.

**Pivalyl-CoA Mutase Activity**—The reaction was performed in Buffer A in a total volume of 0.8–1.4 ml containing 600–2500 μg of Gk IcmF or Cm IcmF, 100 μM AdoCbl, 20–200 μM isovaleryl-CoA, and 15 mM MgCl₂ ± 5 mM GTP. For every concentration of substrate, two to six aliquots (200 μl each) were removed and treated as described below.

**Isobutyryl-CoA Mutase Activity**—The reaction was performed in Buffer A in a total volume of 0.8–1.4 ml containing 10–60 μg of Gk IcmF or Cm IcmF, 100 μM AdoCbl, a saturating
IcmF Is a Pivalyl-CoA Mutase

concentration of substrates (600–2000 μM isobutyryl-CoA or n-butryl-CoA), and 10 mM MgCl₂ ± 3–6 mM GTP or ATP. At various time points (0.5–30 min), 200-μl aliquots were removed and quenched with 100 μl of 2 M KOH containing 0.18 mM valeric acid used as an internal standard. Following addition of 100 μl of H₂SO₄ (15%, v/v), the reaction mixture was saturated with NaCl and extracted with ethyl acetate (250 μl). The extract was analyzed directly by GC using a DB-FFAP (30 m × 0.25-mm-inner diameter, 0.25-μm) capillary column (Agilent). A 5-μl sample was injected in the pulsed splitless mode. The oven temperature was initially at 80 °C. Following 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 was as follows: isobutyric acid, 5.85 min; pivalic acid, 5.99 min; n-butyric acid, 6.5 min; isovaleric acid, 6.96 min; and valeric acid, 7.78 min.

IcmF Assays with Alternative Substrates

We used an HPLC-based assay to evaluate whether 3-hydroxybutyryl-CoA can be converted by IcmF to 2-hydroxyisobutryl-CoA. The assay mixture contained in a total volume of 0.5 ml Buffer A, 10 mM MgCl₂, 100 μM AdoCbl, 2 mM GTP, 0.4–1 mM DL-β-hydroxybutyryl-CoA, and 0.2–0.4 mg Gk IcmF. The reaction was initiated by addition of enzyme, and the reaction mixture was incubated at 37 °C. At different time points (0.5–10 min), 60-μl aliquots were removed, quenched with 2 M TCA (10%, v/v), centrifuged, and subjected to HPLC analysis.

The acyl-CoA esters were separated using an HPLC system equipped with an Alltima HP 5-C₁₈ (250 × 4.6-mm) column (Grace). The detector was set at 254 nm. Solvent A was 50 mM monobasic potassium phosphate, pH 5.4. Solvent B was prepared as follows. 500 ml of MeOH was added to 500 ml of 100 mM monobasic potassium phosphate, pH 5.4 (to give a final concentration of 50 mM potassium phosphate and 50% MeOH). Initial conditions used for separation were 10% solvent B and a flow rate of 1.0 ml/min. Between 5 and 30 min, solvent B was increased to 100% and then held at 100% solvent B for 5 min. At 36 min, solvent B was decreased to 10% and held for 10 min at that composition to equilibrate the column between injections. Under these conditions, the retention time for 3-hydroxybutyryl-CoA was 20.1 min. An authentic standard of 2-hydroxyisobutyryl-CoA was not available, but based on the known performance of the C₁₈ column, branched acyl-CoAs elute earlier than linear isomers.

Enzyme-monitored Turnover of IcmF

Changes in the spectra of Gk IcmF-bound AdoCbl were monitored by UV-visible spectroscopy at 24 °C in Buffer A containing 5–15 mM MgCl₂. Substrates (final concentration of 0.5–4 mM) were added to 20–65 μM apolcmF loaded with 1 or 2 eq of AdoCbl. To check the influence of the Meal domain on catalytic turnover, the reaction was also supplemented with 1–5 mM GTP or ATP. The amount of cob(I)alamin formed under steady-state turnover conditions was calculated from the decrease in absorbance at 525 nm upon substrate addition using a value of Δε₅₂₅nm of −4.8 mm⁻¹ cm⁻¹ (14). During the course of the reaction, AdoCbl was gradually converted to enzyme-bound aquacobalamin (OH₂Cbl) as indicated by the appearance of a 350 nm absorption peak. The increase at 350 nm was fitted to a single exponential function, \[ A = A₀ + A₁(1 − e^{−kt}) \] where A is the absorbance at 350 nm, b is the observed rate constant for inactivation, A₀ is the initial absorbance at 350 nm, t is time in minutes, and A₁ is the amplitude.

Enzyme-monitored Turnover of Gk IcmF under Anaerobic Conditions

To assess the effect of oxygen on enzyme inactivation, enzyme-monitored turnover experiments were performed under anaerobic conditions. For this, Buffer A containing 10 mM MgCl₂ was bubbled with N₂ for 3 h before use and introduced into an anaerobic chamber (containing <0.3 ppm O₂). Stock solutions of AdoCbl, n-butyryl-CoA, isobutyryl-CoA, and isovaleryl-CoA were prepared in the chamber using anaerobic buffer. The enzyme solution was deoxygenated by blowing a stream of N₂ over its surface at 4 °C for 40 min. UV-visible spectra were collected using a Mikropack DH-2000 UV-visible light source connected with fiber optics to a cuvette holder inside the glove box.

HPLC Characterization of Inactivation Products

A solution containing 64 μM Gk holo-IcmF (containing 2 eq of AdoCbl) in Buffer A and 10 mM MgCl₂ ± 5 mM GTP was incubated with 1 mM isobutyryl-CoA at 37 °C in the dark. At various times (0–60 min), 15-μl aliquots were removed and immediately quenched with 60 μl of 0.5% trifluoroacetic acid (TFA). The aerobic inactivation products of AdoCbl, 5′-deoxyadenosine and OH₂Cbl, were monitored by HPLC using an Alltima HP 5-μm C₁₈ (250 × 4.6-mm) column (Grace). All steps of sample preparation and HPLC analysis were performed in the dark. Initial buffer conditions were 92% Solvent C (0.1% TFA in water) and 8% Solvent D (0.1% TFA in acetonitrile) at a flow rate of 1.0 ml/min. Between 10 and 35 min, Solvent D was increased to 32%. Between 35 and 36 min, Buffer D was decreased to 8% and held for 5 min at that composition to equilibrate the column between injections. 50 μl of the sample was injected, and elution was monitored at 254 and 350 nm. Under these conditions, the following retention times were obtained: 6.92 min for 5′-deoxyadenosine, 22.77 min for OH₂Cbl, and 29.27 min for AdoCbl. The control reaction was performed in the absence of isobutyryl-CoA. Calibration curves were generated for all three compounds prepared and treated similarly to the assay samples. An extinction coefficient of ε₅₂₀nm = 15 mm⁻¹ cm⁻¹ was used to estimate the concentration of 5′-deoxyadenosine (17). The data were well fitted by a single exponential function for the disappearance of AdoCbl and appearance of 5′-deoxyadenosine and OH₂Cbl. To improve recovery of OH₂Cbl, proteinase K (Roche Applied Science) was used to digest the protein sample for 1–2 h at 37 °C before precipitation with acid.

Bioinformatics Analysis

Operon and regulon browsers on the Microbes Online website were used for the elucidation of functional predictions for the genes of interest (18).
Gene Neighborhood Analysis for icmF—In several bacteria, the icmF gene is located in the same operon with or in close proximity to genes encoding enzymes involved in fatty acid metabolism (3). For example, enzymes found in the operon with icmF in several bacteria (Bacillus selenitireducens, Lysinibacillus sphaericus, Anoxybacillus flavithermus, Bacillus megaterium, Bacillus halodurans, Bacillus pseudofirmus, Bacillus coagulans, Bacillus sp. NRRL B-14911, Geobacillus sp. WCH70, and Brevibacillus brevis) are annotated as enzymes in the mother cell metabolic gene (mmg) operon, which has been described in Bacillus subtilis (19) (Fig. 5). Three genes in this operon are annotated as mmgA (acyl-CoA transferase), mmgB (3-OH-butyryl-CoA dehydrogenase), and mmgC (acyl-CoA dehydrogenase) (19). A similar set of enzymes inRalstonia eutropha encoded by the H16_A0459–H16_A0464 operon allows growth on long-chain fatty acids. The absence of this operon together with the H16_A1526–H16_A1532 operon renders R. eutropha unable to grow on plant oils or long-chain fatty acids as a carbon source (20). The gene acdA from Streptomyces coelicolor and Streptomyces avermitilis is homologous to mmgC is an acyl-CoA dehydrogenase and plays a role in the catabolism of branched-chain amino acids (21). Finally, the presence of the rpoE gene (which encodes the σ subunit of RNA polymerase) just downstream of icmF strongly suggests that IcmF is linked to fatty acid metabolism (22) (Fig. 5).

Alternative Substrates for IcmF—Based on the above gene neighborhood analysis, we sought to assess whether IcmF plays a role in the metabolism of branched fatty acids and can catalyze the isomerization of substrates other than isovaleryl-CoA and n-butyryl-CoA. We decided to use isovaleryl-CoA, a building block for iso branched fatty acids, and 2-methylbutyryl-CoA, a building block for anteiso branched fatty acids, as potential substrates for IcmF. Isovaleryl-CoA and 2-methylbutyryl-CoA are synthesized from the branched-chain amino acids leucine and isoleucine, respectively, in reactions catalyzed by the branched-chain ketoacid dehydrogenase complex (21).

Gk IcmF was mixed with isovaleryl-CoA in the presence of AdoCbl and GTP/MgCl₂, and the products were hydrolyzed and analyzed by GC. A time-dependent decrease in the isovaleric acid peak at 6.96 min was accompanied by the appearance of a peak at 5.99 min, suggesting conversion of isovaleryl-CoA to a new compound (Fig. 6). The expected product of AdoCbl-dependent 1,2 rearrangement of isovaleryl-CoA (or 3-methyl-butyryl-CoA) is pivaloyl-CoA (or 2,2-dimethylpropionyl-CoA) (Fig. 1) (1). The retention time of a standard pivalic acid sample (5.99 min) exactly coincided with that of the product formed from isovaleryl-CoA (Fig. 6). The isomerization of isovaleryl-CoA to pivaloyl-CoA by both Gk IcmF and Cm IcmF is slow in comparison with the isomerization of n-butyryl-CoA to isobutyryl-CoA (Table 1). The Kₘ value for isovaleryl-CoA for Gk IcmF is 62 ± 8 μM, and the specific activity of the Gk IcmF with isovaleryl-CoA is 0.021 ± 0.004 μmol min⁻¹ mg⁻¹, which is ~150-fold lower than the activity with n-butyryl-CoA (3.25 ± 0.35 μmol min⁻¹ mg⁻¹). With the Cm IcmF, an ~2200-fold difference in the specific activities was obtained with the two substrates (Table 1).

Isomerization of valeryl-CoA is expected to produce 2-methylbutyryl-CoA. However, consumption of valeryl-CoA was not observed in the presence of IcmF, AdoCbl, and GTP/MgCl₂ in the GC-based assay (data not shown). Because HCM (5, 23) from Methylibium petroleiphilum is very similar to the stand-alone ICM from Streptomyces cinnamonensis and catalyzes the interconversion of 3-hydroxybutyryl-CoA and 2-hydroxy-
isobutyryl-CoA (5, 24), we decided to examine their substrate specificity overlap. However, conversion of DL-3-hydroxybutyryl-CoA to 2-hydroxyisobutyryl-CoA was not observed as judged by an HPLC-based assay (data not shown).

Absorption Spectrum of Gk IcmF during Steady-state Turnover—The binding sites for AdoCbl in the Gk IcmF dimer are nonidentical and exhibit an ~25-fold difference in affinities ($K_{D1} = 0.08 \pm 0.01 \mu M$ and $K_{D2} = 1.98 \pm 0.4 \mu M$) (3). For enzyme-monitored turnover experiments, Gk IcmF was reconstituted with 1 eq of AdoCbl to avoid the presence of free cofactor. The absence of free AdoCbl was confirmed by analyzing the spectrum of the flow-through obtained upon concentrating the reaction mixture using a Microcon 30 kDa concentrator. Addition of isobutyryl-CoA to 2-hydroxyisobutyryl-CoA was not observed as contributors to the observed inactivation (data not shown). Protein stability, the presence of metal ions (K and Ca$^{2+}$), reductants (DTT and tris(2-carboxyethyl)phosphine hydrochloride), and different buffers (50 mM sodium phosphate, pH 7.5, 100 mM NaCl; 50 mM potassium phosphate, pH 7.5, 100 mM KCl; and 50 mM HEPES, pH 7.5, 100 mM NaCl) were assessed and ruled out as contributors to the observed inactivation (data not shown).

Nucleotides (ATP and GTP) decreased the activity of Gk IcmF (Fig. 9A). Rapid oxidation of cob(II)alamin to OH2Cbl was observed during reaction of holo-IcmF (with 1 eq; 41 $\mu M$ AdoCbl bound) with 1.4 mM isobutyryl-CoA in Buffer A with 5 mM MgCl$_2$ at 24 °C in the dark. The spectra were recorded between 0 and 60 min. Inset, the time-dependent increase at 350 nm was fitted to a single exponential function in the absence of nucleotides (filled circles) ($k_{obs} = 0.11 \pm 0.01$ min$^{-1}$) or in the presence of 5 mM GTP (open circles) ($k_{obs} = 0.063 \pm 0.002$ min$^{-1}$) or 5 mM ATP (triangles) ($k_{obs} = 0.10 \pm 0.01$ min$^{-1}$).

Under standard in vitro assay conditions, Gk IcmF exhibits a linear reaction time course for only 40–60 s before the activity plateaus (Fig. 9A). The specific activity with isobutyryl-CoA (1.1 ± 0.1 $\mu$mol min$^{-1}$mg$^{-1}$) was calculated using the linear portion of the curve and was comparable with values obtained previously in the coupled enzyme assay (3). When n-butyryl-CoA was used as a substrate, a specific activity of 3.25 ± 0.35 $\mu$mol min$^{-1}$mg$^{-1}$ was obtained. With both isobutyryl-CoA and n-butyryl-CoA, inactivation of Gk IcmF resulted in termination of the reaction in ~6–10 min during which time only ~7% of substrate was consumed (Fig. 9A). Very similar behavior was also observed with Cm IcmF (data not shown). Protein stability, the presence of metal ions ($K^+$ and Ca$^{2+}$), reductants (DTT and tris(2-carboxyethyl)phosphine hydrochloride), and different buffers (50 mM sodium phosphate, pH 7.5, 100 mM NaCl; 50 mM potassium phosphate, pH 7.5, 100 mM KCl; and 50 mM HEPES, pH 7.5, 100 mM NaCl) were assessed and ruled out as contributors to the observed inactivation (data not shown). Nucleotides (ATP and GTP) decreased the activity of Gk IcmF (Fig. 9A) as reported previously with GDP and GTP (3). With isovaleryl-CoA, the Gk IcmF reaction was almost completely inhibited after 25–30 min (Fig. 9B). However, in the presence of GTP, the enzyme was inactivated to a lesser extent in comparison with the reaction without GTP (Fig. 9B). Similar behavior was seen for Cm IcmF (data not shown). Interestingly, for the first 2 min, no difference was seen in the isovaleryl-CoA/pivalyl-CoA mutase activity ± GTP (Fig. 9B).

Loss of 5’-Deoxyadenosine Leads to Inactivation of IcmF—The steady formation of OH$_2$Cbl during IcmF turnover under aerobic conditions suggests that inactivation might result from oxidative interception of cob(II)alamin (27). An alternative possibility is that inactivation is associated with loss of 5’-de-
oxyadenosine from the active site, and the uncoupled cob(II)alamin is subsequently oxidized to OH$_2$Cbl. To distinguish between these possibilities, enzyme-monitored turnover experiments were conducted under anaerobic conditions (Fig. 10A). Addition of $n$-butyryl-CoA to holo-IcmF resulted in formation of cob(II)alamin, which was not further converted to OH$_2$Cbl even after 60 min (Fig. 10A).

Next, we compared the time courses of the reaction catalyzed by Gk IcmF with $n$-butyryl-CoA under aerobic and anaerobic conditions (Fig. 10B). Under anaerobic conditions, the reaction exhibited a linear dependence for a longer time period (2 versus 1 min) but eventually ceased after 10 min. Because spectroscopic analysis of the reaction time course shows that OH$_2$Cbl was observed, B, comparison of time courses for the reaction catalyzed by Gk IcmF at 37 °C under aerobic (circles) and anaerobic (triangles) conditions in the presence of GTP. The aerobic and anaerobic assay mixtures in Buffer A with 15 mM MgCl$_2$ contained 40 μg of IcmF, 100 μM AdoCbl, and 1.5 mM isovaleryl-CoA with (white circles) or without 5 mM GTP (black circles) at 37 °C. Aliquots of the reactions were removed at different time points and analyzed by GC as described under “Experimental Procedures.” Data represent the average of three independent experiments.

Characterization of Inactivation Products by HPLC—The reaction of 64 μM Gk holo-IcmF (containing 1 eq of bound AdoCbl) with 4.8 mM n-butyryl-CoA in Buffer A with 5 mM MgCl$_2$ under anaerobic conditions at 24 °C in the dark. Spectra were recorded at time 0 (black), immediately after addition of substrate (light gray), and 60 min after addition of substrate (dark gray). Formation of cob(II)alamin (480 nm peak) without further conversion to OH$_2$Cbl was observed.

IcmF Is a Pivalyl-CoA Mutase

FIGURE 9. Effect of nucleotides on time course of reactions catalyzed by IcmF. A, time course of the isobutyryl-CoA mutase reaction catalyzed by Gk IcmF at 37 °C. The assay mixture in Buffer A with 10 mM MgCl$_2$ contained 40 μg of holo-IcmF, 100 μM AdoCbl, 1.5 mM isobutyryl-CoA, and either no nucleotides (black circles), 6 mM ATP (triangles), or 3 mM GTP (open circles). B, time course of the isovaleryl-CoA mutase reaction catalyzed by Gk IcmF. The reaction mixture in Buffer A with 15 mM MgCl$_2$ contained 2500 μg of IcmF, 100 μM AdoCbl, and 1.5 mM isovaleryl-CoA with (white circles) or without 5 mM GTP (black circles) at 37 °C. Aliquots of the reactions were removed at different time points and analyzed by GC as described under “Experimental Procedures.” Data represent the average of three independent experiments.

FIGURE 10. Inactivation of Gk IcmF under anaerobic conditions. A, spectral changes upon incubation of 33 μM holo-IcmF (containing 1 eq of bound AdoCbl) with 4.8 mM n-butyryl-CoA in Buffer A with 5 mM MgCl$_2$ under anaerobic conditions at 24 °C in the dark. Spectra were recorded at time 0 (black), immediately after addition of substrate (light gray), and 60 min after addition of substrate (dark gray). Formation of cob(II)alamin (480 nm peak) without further conversion to OH$_2$Cbl was observed. B, comparison of time courses for the reaction catalyzed by Gk IcmF at 37 °C under aerobic (circles) and anaerobic (triangles) conditions in the presence of GTP. The aerobic and anaerobic assay mixtures in Buffer A with 15 mM MgCl$_2$ contained 40 μg of IcmF, 2 mM $n$-butyryl-CoA, and either no nucleotides (solid symbols) or 4.3 mM GTP (open symbols). The data represent the mean ± S.D. of three independent experiments.

FIGURE 11. Formation of OH$_2$Cbl and 5′-deoxyadenosine during enzyme-monitored turnover. Holo-IcmF (64 μM IcmF active site concentration containing 2 eq of AdoCbl) was mixed with 1.5 mM isobutyryl-CoA in Buffer A at 37 °C. All manipulations with the samples and HPLC were performed in the dark. The decay of AdoCbl (open triangles) and the appearance of OH$_2$Cbl (solid triangles) and of 5′-deoxyadenosine (open circles) were monitored over 50 min. As a control for AdoCbl stability during sample handling, the analysis was repeated without addition of isobutyryl-CoA (open squares). The data were fitted by a single exponential function for the disappearance of AdoCbl and appearance of 5′-deoxyadenosine and OH$_2$Cbl. 0.01 min$^{-1}$ corresponded to the rate of AdoCbl disappearance. The concentration of 5′-deoxyadenosine recovered (20.6 ± 0.8 μM) was equal to the concentration of AdoCbl consumed (23 ±
**TABLE 2**

**Kinetics of OH$_2$Cbl and 5′-deoxyadenosine formation**

Values represent the average of at least two independent experiments.

| Nucleotide | $k_{obs}$ | Concentration | $k_{obs}$ | Concentration | $k_{obs}$ | Concentration |
|------------|----------|---------------|----------|---------------|----------|---------------|
|           | min$^{-1}$ | μM           | min$^{-1}$ | μM           | min$^{-1}$ | μM           |
| None      | 0.4 ± 0.04 | 23 ± 1        | 0.23 ± 0.02 | 4.34 ± 0.01 | 0.32 ± 0.01 | 20.6 ± 0.8   |
| GTP       | 0.37 ± 0.04 | 20.65 ± 1.02 | 0.22 ± 0.04 | 5.9 ± 1.4   | 0.35 ± 0.05 | 19.9 ± 1.3   |

**FIGURE 12. Multiple sequence alignment of IcmFs and MeaB from *M. extorquens* showing base specificity loop NKX(D/E).** Accession numbers are as follows: G. kaustophilus, YP_149244; C. metallidurans CH34, YP_582365; R. eutropha H16, YP_724799; Frankia alni, YP_716016; Nocardiia farcinica, YP_117245; B. coagulans, ZP_01696637; Thauera sp., ZP_02841697; Rubrivivax gelatinosus, ZP_00249291; and MeaB, AAL67727. (D/E) substitution by Glu (E) is indicated by an asterisk. All accession numbers are from the NCBI protein database.

1 μM. In contrast, the rate of OH$_2$Cbl formation ($k_{obs} = 0.23 ± 0.02$ min$^{-1}$) was slower, and only 4.34 ± 0.01 μM OH$_2$Cbl was recovered (Table 2). Treatment of inactivated enzyme with proteinase K increased OH$_2$Cbl recovery only marginally (6.3 ± 0.2 μM). The reason for the low yield of OH$_2$Cbl at one site (66% of the AdoCbl at one site) is presently not clear. To ensure that AdoCbl is not cleaved non-enzymatically to 5′-deoxyadenosine during sample preparation and/or chromatographic separation, a control reaction was performed in which substrate was omitted from the reaction mixture (Fig. 11).

IcmF appears to show half-of-sites activity because reconstitution with 1 versus 2 eq of AdoCbl resulted in the same steady-state concentration of cob(II)alamin (data not shown). Surprisingly, in the inactivation experiments, not all the AdoCbl bound to Gk IcmF was converted to OH$_2$Cbl and 5′-deoxyadenosine. Instead, ∼66% of the AdoCbl at one site (i.e., 32 μM) was converted to 5′-deoxyadenosine; this corresponded to the concentration of cob(II)alamin (∼21 μM) under steady-state turnover conditions. This provides further evidence for half-of-sites activity in Gk IcmF. It is unclear why complete inactivation at one of the two active sites was not observed.

**ATPase Activity of IcmF**—In the Mea domains of many IcmFs, the base specificity loop motif NKXD is modified to NKXE (3). In the Gk IcmF, the NKXE sequence is present, whereas in the Cm IcmF, the sequence is NKXD (Fig. 12). The Lys residue in the NKXD motif interacts via hydrophobic interactions with the plane of the guanine ring, whereas the Asp coordinates two nitrogen atoms in the purine ring. Gk IcmF catalyzes the hydrolysis of both GTP and ATP (Table 3). The kinetic parameters for IcmF measured in the presence of various nucleotides are comparable: $k_{cat}$ with ATP is 19 ± 1 min$^{-1}$, and $k_{cat}$ with GTP is 10 ± 1 min$^{-1}$. Although the $K_m$ for ATP is high (1290 ± 300 μM) relative to that for GTP (51 ± 3 μM), the concentration of ATP (3–5 mM) is higher than that of GTP (1 mM) in bacterial cells (28).

To rule out the possibility that the ATPase activity of Gk IcmF is due to a contaminant that co-purifies with the mutase, we first tested the effect of introducing the K213A mutation in the GtgGaGKSS sequence of the P-loop motif, which is important for phosphate binding (29). The K213A Gk IcmF mutant was devoid of both GTPase and ATPase activities. Notably, the mutase activity of the K213A mutant was similar to that of wild-type protein (0.6 μmol min$^{-1}$ mg$^{-1}$ with isobutryl-CoA). Furthermore, in the presence of 3 mM AMPPNP, the $k_{cat}$ for the GTPase activity was inhibited ∼3.6-fold to 2.7 ± 0.14 min$^{-1}$. Taken together, the above results are consistent with the ATPase and GTPase activities being intrinsic to Gk IcmF.

Although it has been suggested that replacement of Asp by Glu in the NKXD motif has no effect on nucleotide specificity (30), our results suggest the contrary. Thus, in Cm IcmF, the base specificity loop sequence is NKFD, and although this protein does not exhibit ATPase activity, it is an active GTPase ($k_{cat}$ = 18 ± 1.3 min$^{-1}$ and $K_{GTP} = 40 ± 8$ μM). These results provide an interesting illustration of a G-protein losing its specificity due to a single substitution in the NKXD motif.

**DISCUSSION**

Previously, it was believed that ICM-like activity was restricted to the *Streptomyces* genus where it is involved in monensin A and B production (4). Our discovery of Icm activity in the IcmF fusion protein, which is widely distributed in bacteria, suggests its involvement in metabolic processes beyond polyketide synthesis (3). In our efforts to elucidate the possible roles of IcmF in bacterial metabolism, we noticed that *icmF* genes co-localize with genes encoding enzymes involved in β-oxidation of fatty acids. Subsequently, we probed branched organic acids, which are synthesized from the branched amino acids valine, leucine, and isoleucine, as substrates for IcmF. This in turn led to the discovery of a new IcmF activity, i.e., isomerization of isovaleryl-CoA/pivalyl-CoA (Figs. 1 and 6).

Although AdoCbl-dependent pivalyl-CoA mutase activity has been predicted to exist (10, 31), an enzyme with this catalytic activity has not been identified previously. In this study, we report that IcmFs from G. kaustophilus and C. metallidurans can convert isovaleryl-CoA to pivalyl-CoA (Fig. 1). Depending on the organism, the isovaleryl-CoA/pivalyl-CoA mutase activity of IcmF was ∼150–2,200-fold lower than the conversion of n-butryl-CoA to isobutryl-CoA (Table 1). Recently, it was shown that pivalic acid can be incorporated as a starter unit in fatty acids in several bacteria (32). Thus, the production of pivalyl-CoA catalyzed by IcmF might be important in bacteria that use this starter unit for the biosynthesis of branched fatty acids containing a quaternary carbon. Our discovery of the isovaleryl-CoA/pivalyl-CoA mutase activity adds to the growing list of carbon skeleton rearrangements catalyzed by AdoCbl-dependent isomerases (Fig. 1). Thus, it appears that the “mutase core” is quite versatile for two reasons. First, sub-
stitions of a limited number of key active site residues alter substrate specificity, and second, relaxed substrate specificity allows alternative reactions to be catalyzed by the same active site as exemplified by IcmF.

We also report relaxed substrate specificity in the MeaB reaction of Gk IcmF. It has been reported that some G-proteins have either lost or switched nucleotide specificity (33). For example, in centaurin γ-1 GTase, where the sequence of the base specificity loop NKXD is modified to TQDR (34), nucleotide specificity is lacking, and it is described as a general NTPase (34). Proteins belonging to the Ynf family of the Obg family of G-proteins harbor the NKXE motif instead of the NKXD (35). For example, human OLA1, which belongs to the Obg family, hydrolyzes ATP more efficiently than GTP.

In our study, we show that in a subset of the MeaB-dependent enzymes that they catalyze reactions involving radical intermediates under aerobic conditions. However, this comes with a price, i.e., their susceptibility to inactivation (12). Under standard in vitro assay conditions, IcmF is activated quite rapidly with either isobutyryl-CoA or n-butyryl-CoA as substrate (Fig. 9). In contrast, when isovaleryl-CoA is used as substrate, protection, albeit modest, was seen in the presence of GTP (Fig. 9B). The activity of Gk IcmF with isobutyryl-CoA is reduced in the presence of nucleotides (3). This is unexpected because MeaB increases the kcat of MCM 1.8-fold in addition to protecting it from inactivation (14). The rate of IcmF inactivation (0.11 ± 0.01 min⁻¹) in the presence of isobutyryl-CoA is ~14-fold higher than inactivation of MCM in the presence of methylmalonyl-CoA (0.0072 min⁻¹) (14).

Under anaerobic conditions, the IcmF reaction with n-butyryl-CoA was linear for a longer duration than in the presence of oxygen (Fig. 10B). Because OH₂Cbl formation is observed only under aerobic conditions, it argues against an internal electron transfer from cob(I)alamin to the substrate being responsible for inactivation as described for lysine 5,6-aminomutase (26). Because IcmF is also inactivated under anaerobic conditions where OH₂Cbl is not formed, we conclude that inactivation is primarily signaled by the loss of 5'-deoxyadenosine from the active site.

In conclusion, we report that IcmF catalyzes the formation of a pivalyl-CoA from isovaleryl-CoA. There is virtually no information on bacterial metabolism of pivalic acid, which has a mostly anthropogenic origin (10). We suggest that pivalyl-CoA mutase activity of IcmF might be important for biodegradation of branched compounds where pivalic acid is a central intermediate (10, 43). The activity of IcmF would reduce branching of compounds with a quaternary carbon. It will be important to follow the fate of pivalyl-CoA in IcmF-containing bacteria and test whether this compound is incorporated in fatty acids and/or other compounds.

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TABLE 3
Comparison of GTPase and ATPase Activities of Gk IcmF

| Enzyme | kcat | Km/GTP | kcat/Km | kcat | Km/ATP | kcat/Km/ATP |
|--------|------|--------|---------|------|--------|-------------|
| Wild type | 10 ± 2 | 51 ± 3 | (1.96 ± 0.37) x 10³ | 19 ± 1 | 1290 ± 300 | (1.47 ± 0.03) x 10⁴ |

† All experiments were performed in Buffer A with 20 mM MgCl₂ at 37 °C as described under “Experimental Procedures.” Values represent the average of at least five independent experiments. ND, not detected.

‡ When a 40-fold higher concentration of the K213A mutant was used in the assay, GTase/ATPase activity above the detection limit of 5 μM inorganic phosphate released in the malachite green assay was not observed.

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IcmF Is a Pivalyl-CoA Mutase

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