IcmF Is a Fusion between the Radical B$_{12}$ Enzyme Isobutyryl-CoA Mutase and Its G-protein Chaperone

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Coenzyme B$_{12}$ is used by two highly similar radical enzymes, which catalyze carbon skeleton rearrangements, methylmalonyl-CoA mutase and isobutyryl-CoA mutase (ICM). ICM catalyzes the reversible interconversion of isobutyryl-CoA and n-butyryl-CoA and exists as a heterotetramer. In this study, we have identified >70 bacterial proteins, which represent fusions between the subunits of ICM and a P-loop GTPase and are currently misannotated as methylmalonyl-CoA mutases. We designate this fusion protein as IcmF (isobutyryl-CoA mutase fused). All IcmFs are composed of the following three domains: the N-terminal 5′-deoxyadenosylcobalamin binding region that is homologous to the small subunit of ICM (IcmB), a middle P-loop GTPase domain, and a C-terminal part that is homologous to the large subunit of ICM (IcmA). The P-loop GTPase domain has very high sequence similarity to the Methylobacterium extorquens MeaB, which is a chaperone for methylmalonyl-CoA mutase. We have demonstrated that IcmF is an active ICM by cloning, expressing, and purifying the IcmFs from Geobacillus kaustophilus, Nocardia farcinica, and Burkholderia xenovorans. This finding expands the known distribution of ICM activity well beyond the genus Geobacillus.

Isobutyryl-CoA mutase (ICM) is a coenzyme B$_{12}$ (or 5′-deoxyadenosylcobalamin [AdoCbl]-dependent enzyme, which catalyzes the rearrangement of isobutyryl-CoA to n-butyryl-CoA (1–3). This reaction is very similar to that catalyzed by methylmalonyl-CoA mutase (MCM), which is better studied and more widely distributed in nature (4). In both reactions, carbon skeleton rearrangements take place where the carbonyl-CoA substituent and a hydrogen atom on neighboring carbon atoms exchange positions (Fig. 1) (2, 3). The genes encoding ICM were first cloned and sequenced from bacteria to man (7).

A G-protein chaperone, MeaB, shows strong operonic association with MCM, and mutations in the human ortholog, the product of the cbI locus, result in methylmalonic aciduria due to dysfunctional MCM activity (8). MeaB from Methylobacterium extorquens has been characterized most extensively and is a P-loop GTPase (9, 10). Other members of this subfamily include HupB, UreG, and CooC, which are important in the assembly of the following metalloenzymes: nickel hydrogenases (11), urease (12), and CO dehydrogenase (13), respectively. MeaB has been proposed to function in the GTP-dependent assembly of holo-MCM and shown to protect the radical intermediates formed during MCM catalysis from oxidative interference (14). MCM, in turn, influences the GTPase activity of MeaB increasing it by 100-fold. Hence, MCM exhibits GTPase-activating protein activity for MeaB (14, 15). In addition, MCM modulates the affinity of MeaB for nucleotides. The crystal structure of MeaB in the presence of GDP has been solved and confirms that it is a member of the G3E family of GTases but differs from other family members in possessing N- and C-terminal extensions of unknown function (9). Structural insights into the interaction between MeaB and MCM are lacking.
IcmF Is an Isobutyryl-CoA Fusion Protein

In this study, we show that in >70 bacteria ICM is fused to a P-loop GTPase, which is a paralog of MeaB. This fusion protein that we have named IcmF (for ICM-fused) is described as a putative MCM-like protein in the data bases. The misannotation has led to the ascription of this gene product as representing a fusion between MCM and MeaB (16) and to its function in pathways that are unlikely to be correct (17). Using bioinformatics and biochemical approaches, we demonstrate that IcmF is an ICM with ICM and GTPase activities. IcmF represents an important paradigm for elucidating the cross-talk between a mutase and its auxiliary protein during the catalytic cycle.

EXPERIMENTAL PROCEDURES

Materials

AdoCbl, GTP, GMPPNP, GDP, isobutyryl-CoA, and n-butyryl-CoA were purchased from Sigma. Tris-(2-carboxyethyl)phosphine hydrochloride (TCEP) was purchased from Pierce. Butyric, isobutyric, and valeric acids were purchased from Fluka. [14C]CH$_3$-malonyl-CoA (56 Ci/mol) was purchased from Pierce. Butyryl-CoA dehydrogenase from Megasphaera elsdenii was purchased from PerkinElmer Life Sciences. A construct harboring butyryl-CoA dehydrogenase from Megasphaera elsdenii was a generous gift from Donald Becker (University of Nebraska, Lincoln).

Cloning and Expression of IcmF

The icmF gene from three organisms was cloned into the pET-30 Ek/LIC expression vector (Novagen, CA). The genomic DNA of Geobacillus kaustophilus and Burkholderia xenovorans (formerly known as Burkholderia fungorum) were generous gifts from Hideto Takami (Japan Agency for Marine-Earth Sciences, Tokyo, Japan) (18). The genomic DNA clone KN023_G20 in the pTS1 plasmid containing the Nocardiia farcinica icmF gene was obtained from Jun Ishikawa (National Institute of Infectious Diseases, Tokyo, Japan) (19).

The resulting fragment was cloned into pGEM-T easy (Promega, WI) and subsequently used as a template for LIC. The insert was amplified with the following primers for LIC cloning, forward 5'-GAGGAGGCAAGATGCGCATTTACCGTGCGGTT-3' and reverse 5'-GAGGAGGAAGCCCGGTTTACTATCATTTGGCGGTATG-3', and inserted in the pET30 Ek/LIC vector according to the manufacturer’s protocol.

icmF from B. xenovorans—The first round of nested-primer PCR was performed on genomic DNA of B. xenovorans with the following primers: forward 5'-CTGTGACCTTCTCTGCTAGGCGGTT-3' and reverse 5'-CGCGACGGGTGTTGTTGTG-3'. The second round of nested PCRs was performed with primers for LIC: forward 5'-GAGGAGGAAGATGCGCATTTACCGTGCGGTT-3' and reverse 5'-GAGGAGGAGCCCGGTATG-3'. The resulting PCR product was subcloned into the pGEM-T vector (Promega, WI) and used for LIC cloning with the following primers: forward 5'-GAGGAGCGAAGATGCGCATTTACCGTGCGGTT-3' and reverse 5'-GAGGAGGAAGCCCGGTATG-3'. The resulting fragment was cloned into pET30 as a template for LIC cloning, containing three protease inhibitor mixture tablets (Roche Ap-
GTPase Activity of IcmF

The GTPase activity of IcmF (5 μM) was determined in the presence of varying concentrations of GTP (50–5000 μM) at 37°C in 0.4 ml of 50 mM KP, buffer, pH 7.5, 100 mM KCl, and 5 mM MgCl2. For each GTP concentration, aliquots (50 μl) were removed at varying time points (2–60 min), quenched with 2 M trichloroacetic acid (10% v/v), centrifuged, and filtered through a 0.1-μm Ultrafree-MC filter (Millipore) to remove the precipitated protein.

The nucleotides were analyzed by ion exchange chromatography on a μBondapak NH2, 300 × 3.9-mm high pressure liquid chromatography column (Waters). Initial conditions were 100% Buffer B (50 mM monobasic KP, pH 4.5) and 0% Buffer C (800 mM monobasic KP, pH 4.5) and a flow rate of 1.0 ml/min. Between 5 and 20 min, Buffer C was increased to 80% and held at that concentration for 5 min. Between 25 and 26 min, Buffer C was decreased to 0% and held for 10 min at that composition to equilibrate the column between injections. Under these conditions, the retention time for GDP was 9.5 min and for GTP was 13.1 min.

Enzyme Assays

Initially, recombinant IcmF was assayed for MCM activity as described previously using the radioactive assay (21). To monitor IcmF activity, one of two assay methods was used. First, a fixed-time GC/MS-based assay was employed by a modification of a method described previously (1, 22). In this assay, normal and isobutyryl-CoA thioesters were saponified, and the resulting free acids were extracted into ethyl acetate. Product formation was followed in a 200-μl assay mixture containing 50 mM KP, pH 7.5, 100 mM KCl, normal butyryl-CoA or isobutyryl-CoA (0.1 to 1 mM), 50 μM AdoCbl, and 0.5–5 μg of IcmF. The reaction was stopped by the addition of 100 μl of 2 N KOH containing 0.18 mM valeric acid as an internal standard followed by addition of 100 μl of H2SO4 (15%, v/v). In the last step of sample preparation, the reaction mixture was saturated with NaCl and extracted with ethyl acetate (250 μl). An aliquot of the extract (5 μl) was subjected to analysis by GC/MS using a DB-FFFAP 30-m × 0.25-mm inner diameter, 0.25-μm capillary column (Agilent, CA). This column is especially designed for the separation of organic acids without derivatization.

A continuous assay was developed to determine the kinetic parameters for IcmF. In this assay, n-butyryl-CoA, which is produced from isobutyryl-CoA by IcmF, is converted to crotonyl-CoA by butyryl-CoA dehydrogenase (BDH). BDH activity was followed by the decrease in absorbance at 300 nm over 1–2 min upon reduction of ferricenium hexafluorophosphate (Fe3+PF6−) (Δε = 4.3 mM−1 cm−1) (23). BDH is able to use both isobutyryl-CoA and n-butyryl-CoA as substrates but with a preference for the latter. We found that with isobutyryl-CoA, K_m = 311 ± 26 μM and V_max = 1.66 ± 0.06 units (μmol/min)/mg, and with n-butyryl-CoA, K_m = 68 ± 4 μM and V_max = 33 ± 1 units/mg. The reaction mixture for the coupled assay contained the following in a final volume of 200 μl: 2–4 μg of IcmF, 50 mM AdoCbl, 0.2 μg of BDH, varying concentrations of isobutyryl-CoA (10–1000 μM), 250 μM (Fe3+PF6−) ± 1–2 mM GDP, GTP, or GMPPNP in 50 mM NaPi, pH 7.5, 250 mM NaCl. Under these conditions, the consumption of isobutyryl-CoA by BDH is negligible and similar to the background rate observed in the absence of BDH. The dye was preincubated for 3 min at 37°C before adding the substrate. After 1 min of incubation, BDH was added, and the reaction was started 1 min later by the addition of holo-IcmF.

UV-visible Spectroscopy

UV-visible spectra were recorded on a Cary 100 spectrophotometer (Varian, Inc., Walnut Creek, CA). Holo-IcmF (10–12 μM) in 50 mM NaPi, pH 7.5, 0.25 mM NaCl ± 5 mM MgCl2, and 1–2 mM GDP, GTP, or GMPPNP was incubated in the presence of 3–5 mM isobutyryl-CoA at 20°C. The spectra were acquired after 2–5 min of incubation.

Isothermal Titration Calorimetry

The isothermal titration calorimetric experiments were performed as described previously (14, 15). Each experiment was performed in triplicate. IcmF was dialyzed for 10–12 h against 50 mM NaPi, pH 7.5, 0.25 mM NaCl containing 1–2 mM TCEP (Buffer D) before use. The protein (8–24 μM) ± 1–2 mM GDP or GMPPNP in Buffer D was titrated with 30–42 7–9.7-μl aliquots of a 15–20 mM excess solution of AdoCbl at 20°C. The calorimetric signals were integrated, and the data were analyzed with Microcal ORIGIN software using a two-sites binding
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model to determine the thermodynamic parameters associated with AdoCbl binding to IcmF.

**EPR Spectroscopy**

EPR spectra were recorded on a Bruker EMX spectrometer (Bruker Biospin Corp., Billerica, MA), equipped with an Oxford ITC4 temperature controller, a Hewlett-Packard model 5340 automatic frequency counter, and a Bruker gaussmeter. Unless otherwise noted, the following parameters were used: temperature, 100 K; microwave power, 25 milliwatts; microwave frequency, 9.38 GHz; receiver gain, $2 \times 10^5$; modulation amplitude, 10 G; modulation frequency, 100 kHz. Cob(II)alamin was generated by treating a solution of hydroxocobalamin with 4–7 molar excess of TCEP. Formation of cob(II)alamin was followed by UV-visible spectroscopy, and the concentration of the solution was estimated using $\epsilon_{273 \text{ nm}} = 9.2 \text{ mM}^{-1} \text{ cm}^{-1}$.

**Bioinformatics Analysis**

STRING was used to find functional linkages for proteins of interest, as well as gene fusions and gene neighborhoods (24). A protein-protein blast search (ncbi.nlm.nih.gov) was used to perform distant searching of homologs. A multiple sequence alignment and phylogenetic tree were constructed using a stand-alone version of ClustalX version 1.8. Figures with multiple sequence alignments were generated using BOXSHADE 3.21. Phylogenetic analysis was carried out using default parameters in ClustalX. The trees were visualized using TreeView 1.6.6. Operon and regulon browsers on the Microbes on-line web site were used for the elucidation of functional predictions for the genes of interest (25).

**RESULTS AND DISCUSSION**

**Bioinformatics Analysis of IcmF**

Analysis of Mutase Domains—Based on bioinformatics analysis, it was previously concluded that MCM either colocalizes in the same operon with its chaperone, MeaB, or that MeaB is fused to the large subunit of MCM in some bacteria (16). Indeed, the putative fusion protein between MCM and MeaB in *B. xenovorans* was reported to possess MCM activity (16). Our laboratory has been elucidating the influence of MeaB and MCM on the substrate binding and catalytic activities of each other (14). Because the kinetics of a fusion protein are easier to characterize than the stand-alone versions of the component proteins, which interact with varying affinities depending on the ligand, we chose to focus on the putative MCM-MeaB fusion protein. A BLAST search using the fusion protein from *B. xenovorans* (NCBI code YP_556774) as a query sequence resulted in the identification of >70 proteins in bacteria, including the seven proteins that were previously identified as examples of fusions between MCM and MeaB (supplemental Table S1) (16). In the data bases, homologs of this fused protein are annotated as putative MCM-like proteins. However, a careful examination of the domain organization and sequence analysis of the substrate-binding site in the B$_{12}$-dependent isomerase component (Figs. 2–4) suggested that this group of fusion proteins might in fact be misannotated.

Based on the high sequence similarity between MCM and ICM, Robinson and co-workers (2) used the crystal structure of MCM from *Propionibacterium shermanii* to identify residues that might be involved in specific substrate binding in ICM from *S. cinnamonensis*. They identified two key substitutions in the large subunit of MCM, Tyr-89 and Arg-207, which are replaced by Phe-80 and Gln-198 in the large subunit of ICM (Fig. 3). These differences in active site residues can be rationalized based on the structural difference between the respective substrates despite the very similar reactions catalyzed by the two enzymes (Fig. 1). In MCM, the carboxylate group of methylmalonyl-CoA is engaged in electrostatic interactions with the guanidinium group of Arg-207 and the phenolic group of Tyr-89 (Fig. 3). The presence of a methyl group in the ICM substrates instead of the carboxylate is reflected in the loss of the hydrogen bond donating arginine and tyrosine residues. Instead, a glutamine and phenylalanine in ICM substitute for the arginine and tyrosine residues, respectively in MCM (Fig. 3). Apart from these two differences, the remaining residues in the active sites of both mutases are highly conserved.

Multiple sequence alignment of the predicted substrate-binding site in the C termini of all the identified fusion proteins clearly reveals conservation of the Phe and Gln residues (Fig. 4 and supplemental Fig. S1). This analysis strongly suggests that the substrate for the fusion protein is n-butyryl-CoA/isobutyryl-CoA, and hence the fusion protein is predicted to be an ICM and not an MCM. We thus designate this fusion protein as IcmF, for isobutyryl-CoA mutase fused. The IcmF designation for this group of fusion proteins also distinguishes it from the “stand-alone” ICM described for the genus *Streptomyces*.

All IcmFs are predicted to be composed of three domains as follows: the N-terminal AdoCbl binding region that is homologous to the small subunit of ICM, a middle P-loop GTPase domain, and a C-terminal region that is homologous to the large substrate-binding subunit of ICM (Fig. 2). Clear sequence similarities are seen between the AdoCbl binding regions of the large subunit of MCM, the small subunit of ICM (IcmB), and the N-terminal portion of IcmF (Fig. 5). The signature DXHXXG...SXL...GG motif (where X is any amino acid) used for binding B$_{12}$ in the “base-off/His-on” conformation is observed in all three proteins (5). However, in IcmF, this motif is similar but not identical to that seen in ICM and MCM. First, a Gly $\rightarrow$ Ala/Ser change is found in IcmF in the following...
IcmF Is an Isobutyryl-CoA Fusion Protein

Expression and Initial Activity Analysis of IcmF

To test the prediction from bioinformatics analysis that IcmF harbors ICM rather than MCM activity, the icmF gene from three organisms, *G. kaustophilus*, *B. xenovorans*, and *N. farcinica*, were cloned into the expression vector pET30 Ek/LIC. Multiple IcmF-encoding genes were subcloned and purified so one organism could be assessed. Because the fusion protein from *B. xenovorans* was reported to have MCM activity (16), we initially tested the activity of all three IcmFs in the standard radiolabeled assay for MCM (21). However, none of the three IcmFs exhibited detectable MCM activity. On the other hand, all three IcmFs exhibited ICM activity. Two assays have been described for monitoring ICM activity and are based on either gas chromatography (GC) or NMR-based detection of the reactant and product (27). Because the NMR-based method is not amenable for routine enzymatic assays, we used a modification of the previously described GC assay (1, 22) using mass spectrometry (MS) for detection of the reaction components. A specific activity of 0.6 ± 0.04 μmol min⁻¹ mg⁻¹ protein at 37 °C was obtained for the *G. kaustophilus* IcmF.

As an alternative to the gas chromatography-mass spectrometry assay that depends on access to specialized instrumentation, a coupled spectrophotometric assay was developed to measure IcmF activity as described under “Experimental Procedures.” The specific activity determined in the coupled assay for the *N. farcinica* IcmF was 1.1 ± 0.1 μmol min⁻¹ mg⁻¹ and for the *B. xenovorans* IcmF was 0.34 ± 0.04 μmol min⁻¹ mg⁻¹ at 37 °C. A specific activity of 0.75 ± 0.01 μmol min⁻¹ mg⁻¹ protein at 37 °C was measured for *G. kaustophilus* IcmF, which is comparable with the value from the gas chromatography-mass spectrometry assay. In comparison, a *V*ₘₐₓ of 38 ± 3 μmol min⁻¹ mg⁻¹ at 37 °C has been reported for purified stand-alone ICM from *S. cinnamomensis* (2).

The recombinant *G. kaustophilus* IcmF was the most stable and soluble of the three proteins and was further purified to ~95% purity as described under “Experimental Procedures” to perform biochemical and biophysical characterizations. Based on its elution from a calibrated gel filtration column, the *G. kaustophilus* IcmF harbors ICM rather than MCM activity, the icmF gene from three organisms, *G. kaustophilus*, *B. xenovorans*, and *N. farcinica*, were cloned into the expression vector pET30 Ek/LIC. Multiple IcmF-encoding genes were subcloned and purified so one organism could be assessed. Because the fusion protein from *B. xenovorans* was reported to have MCM activity (16), we initially tested the activity of all three IcmFs in the standard radiolabeled assay for MCM (21). However, none of the three IcmFs exhibited detectable MCM activity. On the other hand, all three IcmFs exhibited ICM activity. Two assays have been described for monitoring ICM activity and are based on either gas chromatography (GC) or NMR-based detection of the reactant and product (27). Because the NMR-based method is not amenable for routine enzymatic assays, we used a modification of the previously described GC assay (1, 22) using mass spectrometry (MS) for detection of the reaction components. A specific activity of 0.6 ± 0.04 μmol min⁻¹ mg⁻¹ protein at 37 °C was obtained for the *G. kaustophilus* IcmF.

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IcmF appears to be a dimer with a native molecular mass of ~286 kDa.

**Binding of AdoCbl to IcmF ± Nucleotides**

We investigated the energetics of AdoCbl binding to *G. kaustophilus* IcmF ± nucleotides by Fig. 7 and Table 1. These experiments revealed the presence of two non-equivalent binding sites with an ~9–25-fold difference in affinity for AdoCbl that was influenced by the presence and identity of the guanine nucleotide (Table 1). Binding of AdoCbl to the high affinity site in the absence of nucleotides (\(K_D \approx 81 \pm 14\) nM) is accompanied by a \(\Delta G^\circ \) of ~9.5 ± 0.1 kcal/mol that is enthalpically favored, whereas binding to the low affinity site (\(K_D \approx 2.0 \pm 0.4 \mu\)M) is entropically driven (Table 1). These data suggest a possible difference in the flexibility of the two AdoCbl-binding sites in IcmF. A \(K_{act} \) of 12 ± 2 \(\mu\)M for AdoCbl for ICM from *S. cinnamonensis* has been reported (2).

We next analyzed the influence of nucleotides on cofactor binding. The affinity for AdoCbl for the high affinity site was slightly increased in the presence of GDP (132 ± 9 nM), which resulted from changes in both enthalpic and entropic contributions (\(\Delta \Delta H^\circ \sim 2.5 \text{kcal/mol} \) and \(\Delta \Delta S^\circ \sim 2.8 \text{kcal/mol} \)). GDP did not substantially influence binding of AdoCbl to the second site. Binding of AdoCbl in the presence of GMPPNP, a nonhydrolyzable analog of GTP (Fig. 7 and Table 1), indicates that GTP hydrolysis is not required for binding of AdoCbl to IcmF. GDP decreased by ~2-fold the affinity for AdoCbl to site 1 (\(K_D \approx 154 \pm 71\) nM) and slightly increased the affinity at site 2 (\(K_D \approx 1.3 \pm 0.5 \mu\)M). Changes in both the enthalpic and entropic terms contributed to this change. Although cofactor binding to site 1 is enthalpically driven, it is almost entirely entropically driven at site 2.

**IcmF Is an Active Isobutyryl-CoA Mutase**

Using the coupled assay, we further characterized the kinetic parameters for IcmF from *G. kaustophilus* (Fig. 8A and Table 2). The \(K_m\) value for isobutyryl-CoA was determined to be 20 ± 1 \(\mu\)M and the \(k_{cat}\) to be 3.1 ± 0.1 s\(^{-1}\) in the absence of nucleotides. In comparison, for isobutyryl-CoA a \(K_m\) of 57 ± 13 \(\mu\)M has been reported for ICM from *S. cinnamonensis* (2). Thus, the \(k_{cat}/K_m\) values for the stand-alone *S. cinnamonensis* ICM and *G. kaustophilus* IcmF are 6.8 ± 2.7 \(\times 10^5\) M\(^{-1}\) s\(^{-1}\) (2) and 1.5 ± 0.1 \(\times 10^5\) M\(^{-1}\) s\(^{-1}\) respectively. We note that the activity of the
G. kaustophilus was measured at 37 °C in the coupled enzyme assay, which is significantly lower than the optimal growth temperature (60 °C) for the organism (28). Because purified recombinant IcmF was found to be unstable at higher temperatures, its activity at 60 °C could not be measured. Based on a coefficient of 2 for every 10 °C rise in temperature, we estimate that the $k_{cat}$ for this enzyme might be 4-fold higher at 60 °C.

Surprisingly, the presence of GDP or GTP affected both the $k_{cat}$ and $K_m$ values (Fig. 8A and Table 2). Thus, the presence of nucleotides decreased $k_{cat}$ to 1.6–1.7-fold while increasing the $K_m$ to 2-fold. Consequently, the catalytic efficiency $k_{cat}/K_m$ value of IcmF decreased 3.5- and 4-fold, respectively, in the presence of GDP and GTP.

Absorption Spectroscopy of IcmF

As IcmF like ICM is expected to deploy radical chemistry with AdoCbl (Fig. 1), we analyzed whether the presence of nucleotides affected the cob(II)alamin levels under steady-state turnover conditions (Fig. 8B). In the presence of isobutyryl-CoA, the spectrum of holo-IcmF was a 1:2 mixture of cob(II)alamin:AdoCbl. In the presence of nucleotides, accumulation of cob(II)alamin was diminished to 1:4 (GDP) and 1:9 (GMPNP) (Fig. 8B). These results indicate that the nucleotides influence the steady-state distribution of intermediates, which might be related to their effects on $k_{cat}$.

EPR Spectroscopy

The existence of a biradical intermediate has been demonstrated by EPR spectroscopy for MCM from P. shermanii with cob(II)alamin coupled to the product radical (29). However, an EPR spectrum was not observed when 40 μM holo-IcmF was mixed with 7 mM isobutyryl-CoA and frozen rapidly. Because the cob(II)alamin intermediate is observed by UV-visible spectroscopy (Fig. 8B), the lack of a paramagnetic signal suggests strong coupling between it and the organic radical species in the IcmF active site. This has also been observed with MCM from M. extorquens.4

The EPR spectrum of cob(II)alamin bound to IcmF was recorded (Fig. 8C). Binding of cob(II)alamin by IcmF yields an EPR spectrum that is diagnostic for the presence of an axial nitrogen ligand. Hyperfine coupling between the unpaired electron and the $S = 7/2$ cobalt nucleus results in an eight-line spectrum, which is further split into triplets due to superhyperfine coupling to the $I = 1$ axial nitrogen ligand (Fig. 8C, spectrum 1). The spectra of cob(II)alamin bound to IcmF differs from that of free cob(II)alamin (Fig. 8C, spectrum 4) particularly in the $S$-shaped absorption feature at $g \approx 2.3$ and probably results from immobilization of the cofactor in the active site. When IcmF was reconstituted with cob(II)alamin and 5'-deoxyadenosine in the presence or absence of isobutyryl-CoA (Fig. 8C, spectra 2 and 3), the spectra showed sharpening and resolution of additional hyperfine struc-

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4 D. Padovani and R. Banerjee, unpublished results.
ture in the S-shaped feature was observed. These spectral differences suggest conformational changes that influence the electronic properties of the cob(II)alamin radical.

**GT Pase Activity of IcmF**

Because IcmF possesses an Meal-like domain, it was expected that this protein, like MeaB, can hydrolyze GTP. Hence, the kinetics of GTP hydrolysis catalyzed by apo-IcmF was characterized. A Michaelis-Menten analysis of the data yielded the following parameters: $K_m$ (GTP) = 51 ± 3 μM and $k_{cat}$ = 1.8 ± 0.05 min$^{-1}$ (Fig. 8D). In comparison, MeaB alone exhibits a lower intrinsic GTPase activity ($k_{cat}$ = 0.039 ± 0.003 min$^{-1}$), which is increased ~100-fold in the presence of MCM (15).

**Meal Domain of IcmF Is Distinct from MeaB**

The phylogenetic relationship between MeaB, the chaperone for MCM, and the Meal domain of IcmF was evaluated. A dendrogram constructed from the analysis of MeaB and Meal sequences found in the same organisms reveal that the two gene groups cluster separately (Fig. 9). MeaB and Meal are thus paralogs that have evolved to serve specific partner proteins, i.e. MCM and ICM.

The observation of a Meal domain in IcmF raises the obvious question of whether Meal chaperones also exist for stand-alone ICMs. Indeed, as discussed below, analysis of genomic sequence reveals that two Meal-like proteins are found in bacterial genomes, one associated with MCM (MeaB) and the other with ICM (Meal). The diversification of the G domain sequences within each subgroup strongly suggests that the Meal-like domain of IcmF is evolutionarily distinct from MeaB related to MCM.

**Identification of Stand-alone ICMs That Do Not Belong to the Genus Streptomyces**

To investigate the relationships between the chaperones for ICM versus IcmF, we analyzed other bacterial genomes for the presence of stand-alone ICMs and Meals. In our search we assumed the following: (i) the icmA and icmB genes are not necessarily located close to each other, and (ii) the amino acid substitutions corresponding to Phe-80 and Gln-198 in the S. cinnamonensis sequence are always found...
in the large subunit of ICM. A BLAST search using both sub-units of the stand-alone ICM from S. cinnamonomensis as the query sequence identified several stand-alone ICM sequences, primarily in thermophilic archaea but also in halophilic archaea and in a limited number of bacteria (supplemental Table S2 and supplemental Fig. S2). Furthermore, using the MeaI domain of IcmF as a query sequence revealed that genes encoding stand-alone MeaIs can be associated with either the large or the small subunit of ICM (supplemental Table S2). Interestingly, in several organisms, both ICM subunits are localized in the same operon or are in close proximity, e.g. in Desulfotobacterium hafniense, Archaeoglobus fulgidus, and Symbiobacterium thermodphilum. However, in other organisms, the two subunits are not close to each other in the genome, e.g. in Haloarcula marismortui, Halobacterium sp., and in Natronomonas pharaonis (supplemental Table S2). In some organisms, the gene order in an ICM-encoding operon is the following: Meal, small subunit of ICM, large subunit of ICM, and in others the small subunit and Meal colocalize in an operon whereas the large subunit is independently transcribed.

A phylogenetic tree based on the alignment of stand-alone Meals, the Meal domain of IcmF and the MeaBs associated with MCM, reveals significant overall similarity between these proteins (supplemental Fig. S3). However, a careful examination of all available Meal sequences reveals that this group is evolutionarily distinct from MeaB because they form two separate clusters in the dendrogram. In contrast, MeaIs associated with stand-alone ICMs are closely related to the corresponding domain in IcmF. Hence, the Meal and MeaB are paralogs that probably evolved from a common ancestor and have diverged to support specific B12-dependent isomerases.

Implications of the Presence of IcmF

Gene fusion events occur during evolution resulting in the physical coupling of functionally coupled proteins. It is speculated that gene fusions that facilitate functional interactions between and/or coregulation of proteins might be maintained by selective pressure and are more common than gene fissions (30, 31). In this study, we have characterized IcmF, a protein that likely arose by fusion of three genes encoding the large and small subunits of ICM and the chaperone Meal. Bioinformatics analysis has allowed identification of >70 IcmFs in bacterial and archaeal genomes. However, as noted earlier, all these proteins are incorrectly assigned as representing fusions between MCM and MeaB. There are several reasons that could have led to misannotation of IcmF in the data bases. First, ICM activity was believed to be restricted to the genus Streptomyces, whereas the MCM-catalyzed reaction is important in secondary metabolism and is widely distributed in bacteria. Second, the two signature active site substitutions in the S.
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cinnamonensis ICM was missed in the IcmF sequence (16). The importance of these residues in substrate selectivity was previously demonstrated by mutagenesis studies in which the MCM double mutant, Y89F/R207Q, was designed to mimic the active site residues in ICM (32). In contrast to wild-type MCM, the double mutant bound the ICM substrates, n-butyryl-CoA or isobutyryl-CoA, but instead of catalyzing an isomerization it led to inactivation via an internal electron transfer.

Third, the role for a MeaB-like chaperone protein has only been described so far for MCM and could have contributed to inactivation via an internal electron transfer.

In certain bacteria (e.g. Butyrivibrio fibrisolvens and Streptomyces collinus), acetyl-CoA is converted to butyryl-CoA via four reactions, involving acetyl-CoA acetyltransferase (thiolase), 3-hydroxybutyryl-CoA dehydrogenase, 3-hydroxybutyryl-CoA dehydratase (crotonase), and butyryl-CoA dehydrogenase (33–35).

Subjecting the IcmF sequences to operon analysis reveals that in eight bacterial genomes (Lysinibacillus sphaericus, Bacillus sp., Bacillus halodurans, Bacillus coagulans, Bacillus selenitireducens, Bdellovibrio bacteriovorus, Geobacillus sp., and Anoxybacillus flavithermus) IcmF is located in the same operon with enzymes involved in formation of butyryl-CoA from acetyl-CoA. Based on this analysis, we posit that IcmF is involved in butyryl-CoA, rather than methylmalonyl-CoA metabolism in these bacteria.

Myxalaminids are inhibitors of the eukaryotic electron transfer chain that are produced by the myxobacteria, Myxococcus xanthus and Stigmatella aurantiaca (36). In studies on M. xanthus and S. aurantiaca mutants in which the branched-chain ketoacid dehydrogenase was disrupted (bkd mutants), it was shown that isobutyryl-CoA is incorporated into the final product (37). These results were unexpected because the bkd mutants are unable to form isobutyryl-CoA starter units from valine. The authors suggested that fatty acid degradation by α- and β-oxidation of iso-odd fatty acid could be responsible for isobutyryl-CoA synthesis (37). We speculate that the ICM activity of IcmF found in both these bacteria might play a role in this process instead.

Another interesting implication of our study stems from the identification of stand-alone ICMs in a number of archaea and bacteria (supplemental Table S2 and supplemental Fig. S2). Recently, Fuchs and co-workers (17, 38) have reported the discovery of a novel CO2-fixation pathway in several archaea. They have characterized the 16 enzymes in the 3-hydroxypropionate/4-hydroxybutyrate pathway in Metallosphaera sedula. In this pathway, CO2 molecules are fixed with acetyl-CoA and reductively converted to succinyl-CoA. An intermediate step in this pathway is the conversion of methylmalonate/
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It is interesting how MCM-like enzymes have evolved distinct substrate specificities by virtue of very limited changes in their active site residues. Muller and co-workers (39, 40) described a B12-dependent enzyme that is involved in the pathway of degradation of fuel oxygenates. This enzyme in *Methylbium petroleiphilum* PM1 was shown to convert 2-hydroxyisobutyryl-CoA into 3-hydroxybutyryl-CoA. The remarkable feature of this enzyme is that it resembles ICM and has two subunits, IcmA and IcmB. However, in the active site of IcmA, Phe is substituted by Ile, whereas Gln is conserved (see Fig. 5 in Ref. 39). It is interesting that *M. petroleiphilum* also has a copy of the *icmF* and *mcm* genes (based on amino acid substitutions in the active site sequences). Another example of subtle alterations in substrate specificity is seen in ethylmalonyl-CoA mutase from *Rhodobacter sphaeroides* (41). This enzyme interconverts ethylmalonyl-CoA and methylsuccinyl-CoA. Like MCM, ethylmalonyl-CoA mutase is predicted to have Tyr and Arg residues in the active site. However, to utilize the larger

<table>
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<th>TABLE 2: Kinetic parameters for IcmF</th>
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<td><strong>Nucleotide</strong></td>
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<tr>
<td>$K_a$ (isobutyryl-CoA), $\mu M$</td>
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<td>$k_{cat}$, s$^{-1}$</td>
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<td>$k_{cat}/K_a$ M$^{-1}$ s$^{-1}$</td>
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*Note: All experiments were performed in 50 mM NaPi, pH 7.5, 250 mM NaCl at 37 °C as described under “Experimental Procedures.” The data represent the mean ± S.D. of three independent experiments.*

endlonyl-CoA to succinyl-CoA, which is catalyzed by MCM. The majority of MCMs in bacteria are heterodimers, in which one of the subunits binds the substrate and the cofactor. Although *M. sedula* clearly encodes MCM in its genome, some of the putative mutases in other organisms that were identified as MCMs (see supplemental Table S1 in Ref. 17) are predicted to be stand-alone ICMs based on the Tyr → Phe/Arg → Gln substitutions in their active sites. Thus, in *H. marismortui*, *Halobacterium* sp., and *N. pharaonis* stand-alone ICMs rather than MCMs are predicted to exist, raising questions about the presence of an intact 3-hydroxypropionate/4-hydroxybutyrate pathway in these organisms (supplemental Fig. S2). In contrast, in *A. fulgidus* and *Halorubrum lacusprofundi* both copies of the mcm and icm genes are present. In *M. sedula*, only one copy of MCM is present (supplemental Fig. S2). Fuchs and co-workers (17) noted that in some organisms the enzymes from the first half of the cycle are missing and proposed that in this situation reversal of the second half of the pathway might be important for acetyl-CoA assimilation into succinyl-CoA. Interestingly, the first three reactions of this reverse sequence (acetoacetyl-CoA β-ketothiolase, 3-hydroxybutyryl-CoA dehydrogenase, and crotonyl-CoA hydratase) are identical to those in the acetyl-CoA assimilation pathway described for *Streptomyces coelicolor*, which converts acetyl-CoA to crotonyl-CoA (33). The latter, via the action of crotonyl-CoA reductase, is converted to butyryl-CoA, which is isomerized to isobutyryl-CoA by the action of a stand-alone ICM. Isobutyryl-CoA can be converted to succinyl-CoA. Thus, in organisms lacking enzymes in the first half of the 3-hydroxypropionate/4-hydroxybutyrate pathway, ICM may afford an alternative route for assimilation of acetyl-CoA.

FIGURE 8. Kinetic and spectroscopic characterization of IcmF. A, Michaelis-Menten analysis of the IcmF reaction as determined in the coupled enzyme assay. The specific activity (S.A.) of IcmF, analyzed alone (○) or in the presence of GDP (□) or GTP (■), yielded the kinetic parameters reported in Table 2. B, UV-visible spectra of holo-IcmF under steady-state turnover conditions. Holo-IcmF (10.3 μM, solid line) was incubated at 20 °C for 2 min with 3.4 mM isobutyryl-CoA (dotted line) in the presence of 2 mM GDP (dashed line) or GMPNP (dashed-dotted line). C, EPR spectra of IcmF (28 μM) reconstituted with 20 μM cob(III)alamin (spectrum 1) in the presence of 10 mM 5′-deoxyadenosine (spectrum 2) and 8 mM isobutyryl-CoA (spectrum 3). An EPR spectrum of free “base-on” cob(III)alamin (spectrum 4) is shown for comparison. D, Michaelis-Menten analysis of the GTPase activity of IcmF determined as described under “Experimental Procedures.”
ethymalony-CoA/methylsuccinyl-CoA substrates, it is speculated that a conserved His and Asn in MCM are substituted by ethylmalony-CoA/methylsuccinyl-CoA substrates, it is speculated that a conserved His and Asn in MCM are substituted by

Acknowlegements—We thank Dr. Bruce Palfey (University of Nebraska, Lincoln) for providing the M. xanthus dehydrogenase and for help in the developing of coupled assay and Dr. Donald Becker (University of Nebraska, Lincoln) for providing the M. elsdonii BDH expression construct.

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FIGURE 9. Phylogenetic tree of MeaBs that is located in operons with MCM and Meals that are fused to ICM (IcmF). MeaB-like sequences in the same operon with MCM are as follows: MeaB (AAAL86727), B. halodurans (NP_243820), Frankia alni (YP_715132), G. kaustophilus (YP_119677), Leptospira borgpetersenii (YP_384678), L. borgpetersenii (YP_716016), F. alini (YP_630482), and N. farcinica (YP_148222).

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IcmF Is a Fusion between the Radical B$_{12}$ Enzyme Isobutyryl-CoA Mutase and Its G-protein Chaperone
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