Substrate Specificity of the 3-Methylcrotonyl Coenzyme A (CoA) and Geranyl-CoA Carboxylases from *Pseudomonas aeruginosa*\(^+\)^

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Biotin-containing 3-methylcrotonyl coenzyme A (MC-CoA) carboxylase (MCCase) and geranyl-CoA (G-CoA) carboxylase (GCCase) from *Pseudomonas aeruginosa* were expressed as His-tagged recombinant proteins in *Escherichia coli*. Both native and recombinant MCCase and GCCase showed pH and temperature optima of 8.5 and 37°C. The apparent *K_0.5* values of MCCase for MC-CoA, ATP, and bicarbonate were 9.8 μM, 13 μM, and 0.8 μM, respectively. MCCase activity showed sigmoidal kinetics for all the substrates and did not carboxylate G-CoA. In contrast, GCCase catalyzed the carboxylation of both G-CoA and MC-CoA. GCCase also showed sigmoidal kinetic behavior for G-CoA and bicarbonate but showed Michaelis-Menten kinetics for MC-CoA and the cosubstrate ATP. The apparent *K_0.5* values of GCCase were 8.8 μM and 1.2 μM for G-CoA and bicarbonate, respectively, and the apparent *K_m* values of GCCase were 10 μM for ATP and 14 μM for MC-CoA. The catalytic efficiencies of GCCase for G-CoA and MC-CoA were 56 and 22, respectively, indicating that G-CoA is preferred over MC-CoA as a substrate. The enzymatic properties of GCCase suggest that it may substitute for MCCase in leucine catabolism and that both the MCCase and GCCase enzymes play important roles in the leucine and acyclic terpene catabolic pathways.

In the corresponding bacterial catabolic pathways, terpenes are converted to cis-geranyl coenzyme A (G-CoA) and leucine-isovalerate is converted to isovaleryl-CoA. After four analogous reactions that are common to both pathways, the final products of terpene degradation are acetyl-CoA and 3-oxo-7-methyl-6-octenoyl-CoA, and those of leucine catabolism are acetyl-CoA and 3-oxo-7-methyl-6-octenoyl-CoA. In contrast, GCCase catalyzed the carboxylation of both G-CoA and MC-CoA. GCCase also showed sigmoidal kinetic behavior for G-CoA and bicarbonate but showed Michaelis-Menten kinetics for MC-CoA and the cosubstrate ATP. The apparent *K_0.5* values of GCCase were 8.8 μM and 1.2 μM for G-CoA and bicarbonate, respectively, and the apparent *K_m* values of GCCase were 10 μM for ATP and 14 μM for MC-CoA. The catalytic efficiencies of GCCase for G-CoA and MC-CoA were 56 and 22, respectively, indicating that G-CoA is preferred over MC-CoA as a substrate. The enzymatic properties of GCCase suggest that it may substitute for MCCase in leucine catabolism and that both the MCCase and GCCase enzymes play important roles in the leucine and acyclic terpene catabolic pathways.

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INTRODUCTION

A BglII site, and AtuF4, introducing a KpnI site (see Table S1 in the AtuF2. The using the oligonucleotides LiuB1 and LiuB2, AtuC1 and AtuC2, and AtuF1 and end of liuD in this work are ampicillin at 100 g/ml (Sigma and Merck Co.). Antibiotics used were streptomycin at 200 g/ml.

amino acids was tested as described previously (20), using 0.3% (wt/vol) L-leucine medium (22). Solid media were prepared by adding 1.5% agar. Strains were (Promega), pTrc His2A and -C (Invitrogen), and pCDFDuet-1 (Novagen). The JM101 (22), and P. aeruginosa genmic DNA was used giving the pCFC3- dratase; LiuE, 3-hydroxy-3-methyl-glutaryl-CoA (also proposed as isohexenylglutaconyl-CoA hydratase; LiuA, isovaleryl-CoA dehydro- 

FIG. 1. Participation of GCCase and MCCase in the acyclic monoterpane and leucine catabolic pathways of P. aeruginosa PA01 (1, 10). AtuD, citronellyl-CoA dehydrogenase; AtuC/AtuF, GCCase; AtuE, isohexenylglutaryl-CoA hydraelase; LiuA, isovaleryl-CoA dehydro- gene; LiuB/LiuD, MCCase; LiuC, 3-methylglutaryl-CoA hydraelase; LiuE, 3-hydroxy-3-methyl-glutaryl-CoA (also proposed as 3-hydroxy-3-isohexenylglutaryl-CoA lyase).

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains used in this work are Escherichia coli TOP10 (Invitrogen), E. coli BL21 (22), E. coli JM101 (22), and P. aeruginosa PA01SM (25). Plasmids used were pGEM-T Easy (Promega), pTrcHis2A and -C (Invitrogen), and pCDFDuet-1 (Novagen). The strains were grown at 30°C in Luria-Bertani (LB) medium or in M9 minimal medium (22). Solid media were prepared by adding 1.5% agar. Strains were grown on M9 minimal medium supplemented with 0.075% citronellol (Merek) as the sole carbon and energy source. The growth of strains on branched-chain amino acids was tested as described previously (20), using 0.3% (wt/vol) L-leucine supplemented with l-valine and l-isoleucine at 0.005% each (obtained from Sigma and Merck Co.). Antibiotics used were streptomycin at 200 μg/ml and ampicillin at 100 μg/ml.

DNA manipulation and cloning of the atu and liu genes. Genomic and plasmid DNA extraction, restriction enzyme digestion, and agarose gel electrophoresis were carried out by standard methods (22). P. aeruginosa genomic DNA was used as the template for PCR amplification. For liuD gene amplification the oligonucleotide LiuD1 was used to introduce a 5′ BamHI restriction site upstream of the start codon, and LiuD2 was used to introduce a HindIII restriction site at the 3′ end of liuD. For liuB, atuC, and atuF the amplification strategy was the same, using the oligonucleotides LiuB1 and LiuB2, AtuC1 and AtuC2, and AtuF1 and AtuF2. The atuF gene was also amplified using the oligonucleotides AtuF3, introducing a BglII site, and AtuF4, introducing a KpnI site (see Table S1 in the supplemental material).

PCR amplification was carried out using Platinum Pfx DNA polymerase (Invitrogen) according to the manufacturer’s recommendations. Amplified DNA fragments were cloned into the pGEM-T Easy vector, giving plasmids pGluB, pGluD, pGluTuF, and pGluTuC with the cloned liuD, liuB, atuB, and atuC genes, respectively. Recombinant plasmids were subjected to double digestion at the respective enzyme sites designed in the oligonucleotides described above, and the DNA fragments for the liuD, liuB, and atuC genes were subcloned into the pTrcHis2A plasmid, while the atuC gene was subcloned into the pTrcHis2C plasmid, giving the pTrc-liuD, pTrc-liuB, pTrc-atuC, and pTrc-atuC plasmids. Additionally, the atuC and atuF genes were amplified with the AtuC1-AtuC2 and AtuF3-AtuF4 oligonucleotides, respectively; the resulting fragments were cloned into pGEM-T Easy and subcloned into the pCDFDuet-1 coexpression vector, giving the pCFC3-atuC plasmid. The recombinant plasmids were transferred to electrocompetent E. coli JM101 cells and analyzed by digestion with restriction endonucleases and by DNA sequencing.

Expression, purification, and enzyme reconstitution of recombinant proteins. The plasmids containing the atu/liu genes were transferred to E. coli strain TOP10 or BL21, the cells were cultured on LB medium (400 ml), expression was induced with IPTG (isopropyl-β-D-thiogalactopyranoside) (0.1 mM), and the cells were incubated for an additional 4 h at 37°C with shaking. Cells were harvested by centrifugation at 5,000 × g for 10 min at 4°C. The bacterial pellet was suspended in 10 ml of buffer (50 mM Tris-HCl, pH 7.4) and disrupted by sonication at 4°C. The proteins were purified from crude extracts according to the His-bind purification kit protocol (Novagen). The resin-protein mixture was washed twice with 1× wash buffer, and the protein was eluted using 1 ml of elution buffer containing 100 mM imidazole.

Reconstitution of MCCase and GCCase was carried out using the subunits purified as described above and denaturing with 6 M urea. The α and β subunits (2 mg each) were mixed and renatured by dialysis using a Spectrum 10-kDa molecular-mass-cutoff (Fisher Scientific) membrane in a buffer containing 20 mM K2HPO4, 20% glycerol, and 0.75 mM dithiothreitol (pH 8.0) for 1 h at 4°C, changing the buffer (100 ml) six times.

G-CoA synthesis. G-CoA was synthesized by the mixed anhydride method of Hajra and Bishop (14). Geranic acid (Sigma-Aldrich) (770 μmol) was dried twice with 500 μl of hexamethy geran and then 500 μl of benzene under a gentle N2 stream; the same molar amount of butylated hydroxytoluene dissolved in benzene was added, and 400 μl of oxyl chloride and 800 μl of benzene were added and flushed with N2. The mixture was then incubated at 36°C in a water bath for 1 h, dried under a stream of N2, washed with 200 μl of benzene, and dried. CoA (29 μmol) was dissolved in 400 μl of 0.125 mM NH4HCO3 in water adjusted to pH 8.8 with NH4OH and 0.8 ml of tetrahydrofuran. The mixture was stirred at 36°C in a water bath for 30 min. The reaction was stopped with 20 μl 20% H2O2, and the products were dried under an N2 gas stream. The residue was dissolved in 100 μl 2% HClO4, and the mixture was lyophilized. The lyophilized G-CoA was dissolved in water, and its concentration was determined by the hydroxamate method (19).

Determination of MCCase and GCCase activities. Cultures of the P. aeruginosa PA01 strain were grown with shaking at 30°C in 50 ml of M9 medium with 0.075% of citronellol or 0.3% of leucine as a carbon source for 48 h. Cells were harvested by centrifugation and washed with 50 ml of 100 mM K2HPO4, pH 8.0. Pellets were suspended in 5 ml of the same buffer, disrupted by sonication, and centrifuged for 10 min to 15,000 × g at 4°C to eliminate undisrupted cells and cell debris. The protein content was determined by the Bradford method as described previously (22). Purification of MCCase and GCCase from P. aeruginosa crude extracts was carried out using avidin-agarose resin (Sigma), with equilibration with phosphate-buffered saline (PBS) (pH 7.4) with 1 mg/ml of biotin; the crude extract was added to the resin and incubated for 2 h at 4°C. The mixture was washed with PBS, and biotinylated proteins were eluted with PBS containing 2 mM biotin. MCCase and GCCase activities in the extracts or in purified fractions were measured by the incorporation of radioactivity from 14CO2 into acid-stable, nonvolatile material as previously described (15, 21). The reaction mixture contained 20 mM K2HPO4 (pH 8.5), 10 mM MgCl2, cell extract (300 μg of protein) or 40 μg of purified protein, ATP either at 5 mM or in the range of 0 to 40 μM, NaNHCO3 (specific activity, 1.96 GBq/mmol [53 mCi/mmol]; Amersham) either at 10 mM or in the range of 0 to 10 mM, and 3-MC-CoA (Sigma) or cis-G-CoA (synthesized as described above) either at 100 μM or in the range of 0 to 100 μM, in a total reaction volume of 100 μl. The reaction was started by the addition of NaNHCO3 (prepared as 1:10 NaNHCO3-Na2HCO3 to 10 mM), and the mixture was incubated at 37°C for 10 min. The reaction was stopped by adding 200 μl of 10% HClO4. The contents of the tubes were evaporated to dryness at 90°C, and the residue was suspended in 100 μl of distilled water. Radioactivity was quantified using a liquid scintillation counter (Hewlett-Packard 1600 TR). Non-specific 14CO2 fixation was assessed in the absence of substrate. Specific activity was calculated as dpm of 14CO2 fixed/0.53 μmol · min−1 · g−1 of protein.
Western blot analysis. *Pseudomonas aeruginosa* cell extract samples containing 100 μg of protein or 10 μg of purified proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10%) polyacrylamide gel electrophoresis and electrothermally transferred to nitrocellulose membranes (Amersham Biosciences). MCCase and GCCase α subunits (LiuD and AtuF proteins) were detected by using the avidin-horseradish peroxidase (HRP) conjugate (Bio-Rad) as previously described (1). MCCase and GCCase β subunits (LiuB and AtuC) were detected using polyconval mouse antibodies. Antibodies were obtained from a polyclonal serum after immunization of mice with LiuB or AtuC recombinant protein expressed from the pTrc-luIB and pTrc-atuC plasmids and purified from the cell extracts as described above. The polyclonal serum was obtained from the Iowa State University Hybridoma Facility (www.biotech.iastate.edu/service_facilities/hybridoma.html). The membranes were probed using the polyclonal serum, and the anti-mouse–HRP conjugate was used as the second antibody (donkey anti-mouse immunoglobulin G-HRP; Santa Cruz Biotechnology, Inc.) as indicated by the provider. HRP color development was carried out using 4-chloro-1-naphthol (Sigma) and H₂O₂.

RESULTS AND DISCUSSION
Characterization of MCCase and GCCase from *P. aeruginosa* grown on leucine and citronellol. Previous data from our group suggested that MCCase and GCCase show catalytic activity with both MC-CoA and G-CoA substrates, indicating a possible bifunctional role of these enzymes in both the leucine/isovalerate and acyclic terpene catabolic pathways (1). In this work we found that MCCase or GCCase can be coexpressed from cultures of *P. aeruginosa* but that it was difficult to discriminate whether the enzymes showed bifunctionality over both G-CoA and MC-CoA substrates (1). Similar results were found for *P. citronellolis* (9, 15).

To test whether the native properties of MCCase are conserved after heterologous expression of the recombinant enzyme, the MCCase was also purified and characterized as isolated from its native host, *P. aeruginosa*. As indicated in previous reports, MCCase is induced when *P. aeruginosa* grows on leucine as the sole carbon and energy source (1, 16); thus, MCCase was purified from *P. aeruginosa* cells grown on leucine. With the purified fraction, the optimal pH and temperature for MCCase activity were 8.5 and 37°C, respectively (8), as also found in pea leaf and potato mitochondria (9). This is consistent with the behavior of *P. citronellolis* MCCase and is specific in its ability to carboxylate G-CoA and MC-CoA or that under the conditions used a subunit exchange mechanism between MCCase and GCCase may possibly contribute to a non-specific carboxylase enzyme. This behavior could explain why atuC and atuF mutants are affected in their ability to grow on leucine and why an atuF mutant regains the ability to grow on leucine when it is transformed with the liu cluster (1). Therefore, we decided to carry out the characterization of both enzymes by expressing them in *E. coli*, a bacterium that does not possess the MCCase and GCCase enzymes.

Expression of the MCCase and GCCase from *P. aeruginosa* in *E. coli*. The individual enzyme subunits were expressed in *E. coli* and recovered efficiently, obtaining preparations that were >90% pure (Fig. 2A, lanes 1 to 4). In SDS-PAGE the proteins showed relative molecular masses for the LiuD, LiuB, AtuF, and AtuC subunits of 78, 63, 74, and 63 kDa, respectively (Fig. 2A). The expressed and purified LiuB-His and LiuD-His subunits of MCCase did not support enzyme activity. Several attempts to restore the activity of this enzyme using different renaturation conditions were conducted, without success. However, when the purified α and β subunits were combined, denatured, and then renatured as described above, the active MCCase enzyme was recovered. This result shows that incorporation of a His tag does not disturb the function of MCCase, and therefore this recombinant MCCase enzyme was used for the kinetic characterization as described below. In contrast, this strategy was not successful in generating a functional GCCase enzyme. However, a functional GCCase enzyme was successfully reconstituted when the AtuC and AtuF subunit genes for MCCase and GCCase activities were KMₐ₅₀ of 8.84 and 8.80 μM and Vₐ₅₀ of 591 and 627 nmol/min · mg of protein for MC-CoA and G-CoA, respectively. As mentioned above, the purification method used did not differentiate between GCCase and MCCase activities, and therefore it was not possible to elucidate whether GCCase also carboxylates MC-CoA. In addition, these data suggest that GCCase may be nonspecific in its ability to carboxylate G-CoA and MC-CoA or that under the conditions used a subunit exchange mechanism between MCCase and GCCase may possibly contribute to a non-specific carboxylase enzyme. This behavior could explain why atuC and atuF mutants are affected in their ability to grow on leucine and why an atuF mutant regains the ability to grow on leucine when it is transformed with the liu cluster (1). Therefore, we decided to carry out the characterization of both enzymes by expressing them in *E. coli*, a bacterium that does not possess the MCCase and GCCase enzymes.

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were coexpressed and copurified as described above. The coexpressed AtuF-S protein was found to bind to the AtuC-His subunit and was also copurified (Fig. 2). A similar behavior was observed during the expression of the AccD1-His subunit of acetyl-CoA carboxylase from Corynebacterium glutamicum (12) and the acyl-CoA carboxylase from Streptomyces coelicolor A3(2) (5).

SDS-PAGE analysis of this purified fraction indicated that it contained three major protein bands (Fig. 2A, lane 5). Western blotting analysis with avidin-HRP conjugate showed that the upper band (74 kDa) was a biotinylated protein, and because it corresponded in molecular mass to the biotinylated subunit of the GCCase purified from P. aeruginosa extracts grown on citronellol (Fig. 2B, lanes 5 and 6, respectively), we identified this as the recombinant biotinylated AtuF subunit of the GCCase. Similar Western blot analyses with polyclonal anti-AtuC antiserum identified the lowest band (63 kDa) as the recombinant AtuC subunit, corresponding to the GCCase/H9252 subunit; the molecular mass of this band is similar to that of the AtuC subunit purified from P. aeruginosa extracts grown on citronellol (Fig. 2C, lanes 5 and 6, respectively). Although only a faint signal was identified in the purified LiuB protein sample with the anti-AtuC serum (Fig. 2C, lane 1), it was clear from the difference in signal intensity that this is a cross-reactivity of the antiserum. These results established that after coexpression of the AtuC-His and AtuF-S proteins in E. coli, the GCCase complex was reconstituted, and therefore this complex was used for kinetics characterization. It was found that for both the recombinant MCCase and GCCase enzymes, the optimal pH and temperature were 8.5 and 37°C, respectively (data not shown). These values were identical to those for the native host enzymes and similar to those reported for MCCases from mammalian (18), bacterial (23), and plant (2, 3, 4, 7) sources. Like MCCase from P. citronellolis, MCCase and GCCase from P. aeruginosa are inactivated by temperatures higher than 50°C (15) (see Fig. S2 in the supplemental material), in contrast to MCCases from pea leaves and potato mitochondria, which are stable above that temperature (2).

Kinetic parameters of recombinant MCCase. The dependence of MCCase activity on substrates was tested at the optimal conditions, pH 8.5 and 37°C. A typical sigmoidal behavior was observed with respect to MC-CoA, while no activity was observed when G-CoA was assayed as the substrate (Fig. 3A). In kinetics calculations the values were adjusted to Hill’s equation, showing a correlation coefficient of 0.99 with a Hill’s coefficient of 2.3, which suggested that the enzyme has an oligomeric conformation and could provoke cooperative effects of the substrate. The kinetic constants of this enzyme for MC-CoA were a \( K_{0.5} \) of 9.8 \( \mu \)M and a \( V_{\text{max}} \) of 279 nmol/min·mg of protein; these values are in agreement with values obtained with the MCCase purified from the native host, P. aeruginosa. The catalytic efficiencies (\( V_{\text{max}}/K_{\text{m}} \)) of the two enzyme preparations also were similar (46 and 56, respectively), indicating that the recombinant proteins and the heterologous expression did not affect MCCase functionality, as occurs in other carboxylases (5, 12). On the other hand, the kinetic dependence on the ATP and NaHCO3 substrates showed a sigmoidal response, suggesting an allosteric regulation of MCCase by ATP and NaHCO3 (Fig. 3C and D). The apparent kinetic parameters for ATP were a \( K_{0.5} \) of 13 \( \mu \)M and a \( V_{\text{max}} \) of 356

FIG. 3. Kinetic behavior of recombinantly produced P. aeruginosa MCCase and GCCase enzymes. (A and B) Carboxylase activities of recombinant LiuB/LiuD (A) and AtuC/AtuF (B) proteins, purified and reconstituted as described in Materials and Methods. (C and D) ATP (C) and bicarbonate (D) concentration dependence of the MCCase and GCCase activities. Data given are the average of three determinations; standard deviations of the given values are shown in panel A, and the averages of two determinations with variations of less than 5% of the given values are shown in panels B, C, and D.
nmol/min · mg of protein, and those for NaHCO₃ were a $K_m$ of 0.8 μM and a $V_{max}$ of 178 nmol/min · mg of protein. We conclude, therefore, that the MCCase from *P. aeruginosa* is specific for the MC-CoA substrate.

**Kinetic parameters of recombinant GCCase.** Kinetic parameters for the heterologously coexpressed AtuC/AtuF proteins were also measured at optimal conditions (pH 8.5 and 37°C). Under these conditions, the AtuC/AtuF complex catalyzed both GCCase and MCCase enzymatic activities. In relation to the G-CoA substrate, this enzyme exhibited sigmoidal kinetics, adjusting to Hill’s equation with coefficient of 2.2, but in relation to the MC-CoA substrate, it exhibited Michaelis-Menten kinetics (Fig. 3B). These results indicate that the AtuC/AtuF enzyme is able to utilize both G-CoA and MC-CoA as substrates. The kinetic constants with G-CoA were a $K_m$ of 8.8 μM and a $V_{max}$ of 492 nmol/min · mg of protein, and those with MC-CoA were a $K_m$ of 14 μM and a $V_{max}$ of 308 nmol/min · mg of protein. The catalytic efficiencies for the AtuC/AtuF enzyme were 65 for G-CoA carboxylation and 22 for MC-CoA carboxylation. These results indicate that the AtuC/AtuF enzyme prefers G-CoA over MC-CoA as a substrate, and therefore should be considered a GCCase enzyme. In *P. citronellolis* it has been observed that GCCase is able to carboxylate 5 to 15 different acyl-CoA substrates, including MC-CoA (8). An interesting fact is that the plant GCCase shows a strict substrate preference, carboxylating G-CoA but not MC-CoA (13). This finding suggests that in *P. aeruginosa* GCCase may play a bifunctional role during its participation in both the acyclic terpene and the leucine catabolic pathways. Using G-CoA as the carboxylation substrate, the dependence of enzy-

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