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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 $K_{0.5}$ (affinity constant for non-Michaelis-Menten kinetics behavior) 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 acetoacetate (Fig. 1). After two β-oxidation cycles, 3-oxo-7-methyl-6-octenoyl-CoA yields 3-methylcrotonyl-CoA (MC-CoA), an intermediary of the leucine-isovalerate pathway (Fig. 1). Therefore, the acyclic terpene utilization and leucine-isovalerate pathways converge in the MC-CoA intermediate (1, 10). Two homologous gene clusters that encode the enzymes of the acyclic terpene (*atuABCDEFGH*, for *a*cyclic *t*erpenes *u*tilization) and leucine-isovalerate (*liuRABCDE*, for *l*eucine-*i*sovalerate *u*tilization) catabolic routes have been recently identified in *Pseudomonas aeruginosa* (1, 6, 10, 16) and *Pseudomonas citronellolis* (11). Phylogenetic analysis of the *P. aeruginosa* AtuF α subunit of G-CoA carboxylase (GCCase) suggested that it originated by a horizontal transfer event from alphaproteobacteria to *P. aeruginosa* and may implicate different functions (1).

Key enzymes in both pathways are GCCase, encoded by the *atuC*/*atuF* genes, and MC-CoA carboxylase (MCCase), encoded by the *liuB*/*liuD* genes (Fig. 1). These enzymes are composed of two subunits, i.e., LiuB and AtuC (β subunits) and LiuD and AtuF (α subunits), of their respective MCCase and GCCase enzymes (1, 10, 16). MCCase and GCCase enzymes show two main domains, the acyl-CoA-binding and the carboxybiotin-binding domains, which are implicated in the transfer of a carboxyl group to the acyl-CoA substrate (17). On the other hand, both LiuD and AtuF (α subunits) show four highly conserved domains in the acyl-CoA carboxylases: (i) the ATP-binding site (GGGGKGM), (ii) a CO_2 fixation domain (RDCS), (iii) the catalytic site of the biotin-dependent carboxylase family (EMNTR), and (iv) a biotin-carboxyl carrier domain (AMKM).

It has been suggested that the *P. citronellolis* GCCase carboxylates both G-CoA and MC-CoA, while MCCase carboxylates only MC-CoA (9, 13, 15). Although the genes that encode the enzymes of the acyclic terpene and leucine/isovalerate catabolic pathways in *P. aeruginosa* and *P. citronellolis* have been recently elucidated (1, 10), there is still uncertainty regarding the bifunctionality of the carboxylases involved. Using an indirect method for GCCase and MCCase activity determination (a coupled reaction with pyruvate kinase and lactate dehydrogenase), Förster-Fromme et al. (10) suggested that both GCCase and MCCase showed specific activities for their substrates G-CoA and MC-CoA, respectively. However, data from our group suggest that the two catabolic pathways may share gene products or that some enzymes could show a bifunctional activity (1).

This work was focused on the biochemical characterization of MCCase and GCCase and on elucidation of whether these enzymes can use both MC-CoA and G-CoA as substrates.

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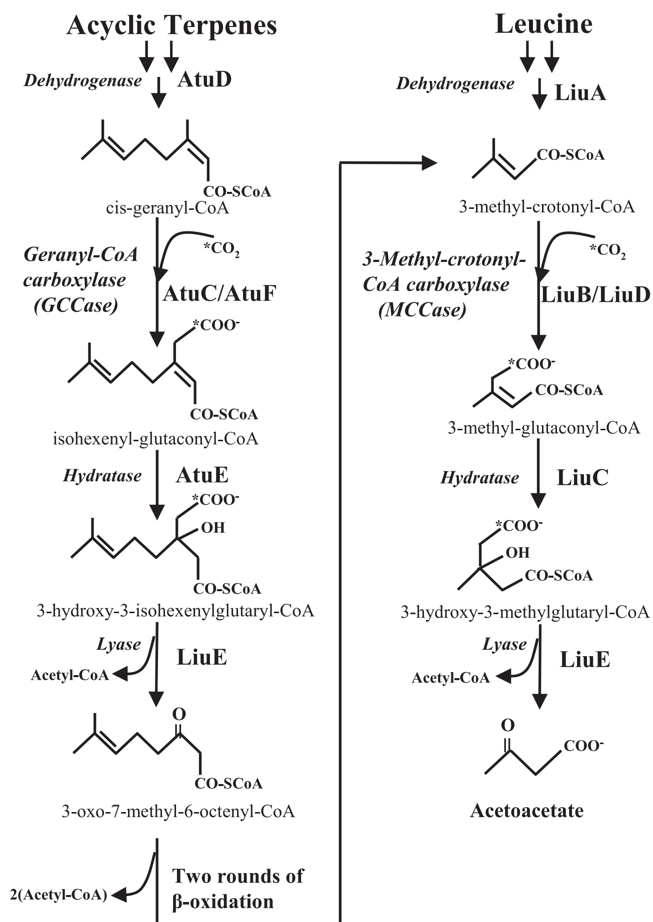

FIG. 1. Participation of GCCase and MCCase in the acyclic monoterpene and leucine catabolic pathways of *P. aeruginosa* PAO1 (1, 10). AtuD, citronellyl-CoA dehydrogenase; AtuC/AtuF, GCCase; AtuE, isohexenylglutaconyl-CoA hydratase; LiuA, isovaleryl-CoA dehydrogenase; LiuB/LiuD, MCCase; LiuC, 3-methylglutaconyl-CoA hydratase; 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* PAO1SM (25). Plasmids used were pGEM-T Easy (Promega), pTrc His2A 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 (Merck) 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 pG*liuB*, pG*liuD*, pG*atuF*, and pG*atuC* with the cloned *liuB*, *liuD*, *atuF*, 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 *liuB*, *liuD*, and *atuC* genes were subcloned into the pTrcHis2A plasmid, while the *atuF* gene was subcloned into the pTrcHis2C plasmid, giving the pTrc-*liuB*, pTrc-*liuD*, pTrc-*atuC*, and pTrc-*atuF* 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-*atuFC* 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 K_2HPO_4, 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 benzene under a gentle N_2 stream; the same molar amount of butylated hydroxytoluene dissolved in benzene was added, and 400 μl of oxalyl chloride and 800 μl of benzene were added and flushed with N_2. The mixture was then incubated at 36°C in a water bath for 1 h, dried under a stream of N_2, washed with 400 μl of benzene, and dried. CoA (29 μmol) was dissolved in 400 μl of 0.125 mM NH_4HCO_3 in water adjusted to pH 8.8 with NH_4OH 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% $HClO_4$, and the products were dried under an N_2 gas stream. The residue was dissolved in 100 μl 2% $HClO_4$, 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* PAO1 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 K_2HPO_4, 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 $^{14}CO_2$ into acid-stable, nonvolatile material as previously described (15, 21). The reaction mixture contained 20 mM K_2HPO_4 (pH 8.5), 10 mM $MgCl_2$, 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 mM, $NaH^{14}CO_3$ (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 $NaH^{14}CO_3$ (prepared as 1:10 $NaH^{14}CO_3$-$NaHCO_3$ to 10 mM), and the mixture was incubated at 37°C for 10 min. The reaction was stopped by adding 200 μl of 6 M HCl, the contents of the tube 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). Nonspecific $^{14}CO_2$ fixation was assayed in the absence of substrate. Specific activity was calculated as dpm of $^{14}CO_2$ fixed/0.53 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ 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) and electrophoretically 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 polyclonal mouse antibodies. Antibodies were obtained from a polyclonal serum after immunization of mice with LiuB or AtuC recombinant protein expressed from the pTrc-*liuB* and pTrc-*atuC* plasmids and purified from the cell extracts as described above. The polyclonal serum was obtained at 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 H_2O_2.

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 by 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 (data not shown). With increasing concentrations of the MC-CoA substrate, a sigmoidal kinetics of MCCase activity was observed (see Fig. S1 in the supplemental material). The kinetic constants for MCCase using MC-CoA as the substrate are a $K_{0.5}$ of 9.2 μM and a V_{max} of 425 nmol/min · mg of protein. Under this condition the MCCase enzyme did not carboxylate the analogous substrate, GC-CoA, indicating that MCCase from *P. aeruginosa* specifically recognizes MC-CoA as its substrate and therefore does not possess GCCase activity (see Fig. S1 in the supplemental material). This is consistent with the behavior of *P. citronellolis* MCCase, which is specific for the MC-CoA substrate and has no detectable GCCase activity (8), as also found in pea leaf and potato mitochondria (2). A difference between the MCCases of *P. aeruginosa* and *P. citronellolis* is the apparent fivefold-higher affinity for the MC-CoA substrate ($K_{0.5}$s of 9.2 μM and 43 μM, respectively). On the other hand, when *P. aeruginosa* was grown on citronellol as the sole carbon source, both the MCCase and GCCase enzymes were expressed (data not shown). Under these conditions MCCase and GCCase were detected and the enzymatic activities displayed similar behavior with both substrates, showing typical sigmoidal behavior with similar kinetic constants (see Fig. S1 in the supplemental material). The kinetic parameters for MCCase and GCCase activities were $K_{0.5}$s of 8.84 and 8.80 μM and V_{max}s 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 nonspecific 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.

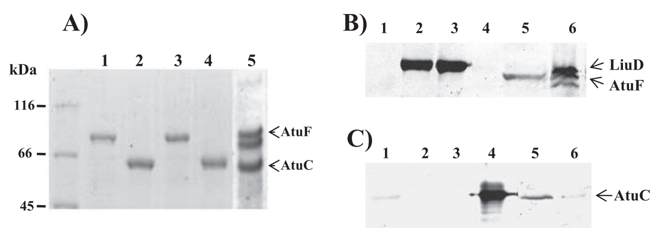

FIG. 2. Electrophoretic analysis of the recombinant heterologous expression of *P. aeruginosa* MCCase and GCCase subunits. (A) Coomassie blue-stained SDS-PAGE analysis of assembled gels of recombinant proteins expressed in *E. coli* and purified using affinity chromatography. Lanes: 1, AtuF-His; 2, AtuC-His; 3, LiuD-His; 4, LiuB-His expressed from pTrcHis-2A vector; and 5, AtuC-His/AtuF-S expressed from pCDFDuet-1 coexpression vector. Molecular mass makers are shown on the left; the protein bands corresponding to AtuC/AtuF are indicated with arrowheads. (B) Western blot analysis of purified recombinant proteins probed with avidin-HRP conjugate to detect biotin-containing proteins. Lanes: 1, LiuB-His; 2, LiuD-His; 3, AtuF-His; 4, AtuC-His; 5, AtuC-His/AtuF-S; and 6, extract from PAO1 culture grown on citronellol. (C) Western blot analysis of purified recombinant proteins probed first with anti-AtuC–His polyclonal antibody and then with anti-mouse–HRP as a secondary antibody. Lanes are the same as in panel B.

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

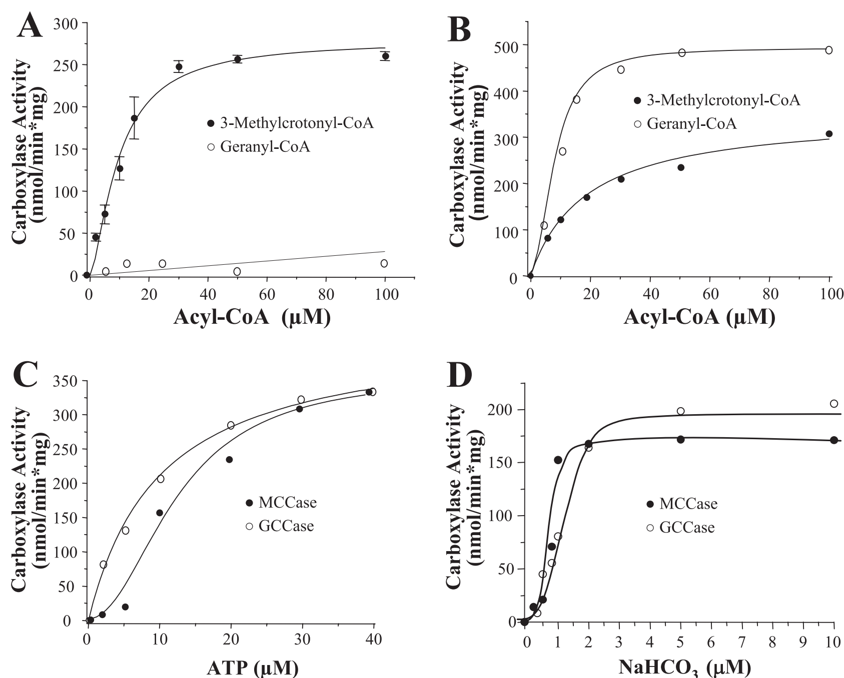

FIG. 3. Kinetic behavior of recombinantly produced *P. aeruginosa* MCCase and GCCase enzymes. (A and B) Carboxylase activities of recombinant LiuB/LiuD (A) and AtuC/AtuF (B) proteins, purified and reconstituted as described in Materials and Methods. (C and D) ATP (C) and bicarbonate (D) concentration 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.

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 β 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 μM and a V_{max} of 279 nmol/min · mg of protein; these values are in agreement with values obtained with the MCCase purified from of the native host, *P. aeruginosa*. The catalytic efficiencies (V_{max}/K_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 $NaHCO_3$ substrates showed a sigmoidal response, suggesting an allosteric regulation of MCCase by ATP and $NaHCO_3$ (Fig. 3C and D). The apparent kinetic parameters for ATP were a $K_{0.5}$ of 13 μM and a V_{max} of 356

nmol/min · mg of protein, and those for $NaHCO_3$ were a $K_{0.5}$ 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 $K_{0.5}$ 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 56 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 enzymatic activity on ATP and $NaHCO_3$ was also tested. In relation to ATP a typical Michaelis-Menten kinetics was observed (Fig. 3C), whereas with $NaHCO_3$ a sigmoidal kinetics was observed (Fig. 3D). The kinetic parameters of GCCase for ATP were a K_m of 10 μM and a V_{max} of 423 nmol/min · mg of protein, and those for $NaHCO_3$ were a $K_{0.5}$ of 1.2 μM and a V_{max} of 210 nmol/min · mg of protein.

The kinetic parameters of both MCCase and GCCase with the cosubstrates ATP and $NaHCO_3$ followed similar tendencies, as could be expected because they have common cosubstrates.

This is the first report showing that MCCase and GCCase display sigmoidal kinetic behavior for their substrates and cosubstrates. These results are interesting because most of the MCCases characterized to date show typical Michaelis-Menten kinetics against their substrates. A sigmoidal kinetic behavior would indicate that the active site is modified by the binding of each substrate, increasing the affinity for the next one, and/or that the binding of substrate promote the oligomerization of the enzyme subunits, favoring their activity. A precedent that supports this finding may be found with pyruvate carboxylase and the MCCases from pea leaf and potato mitochondria, in which the domain arrangement provides a mechanism for allosteric activation (24) and sigmoidal behavior with respect to Mg^{2+} variations (2, 3).

In conclusion, our results indicate that MCCase, encoded by *liuB/liuD* genes, specifically recognizes MC-CoA as its substrate, and the bifunctionality of the GCCase suggests that it may supplant the MCCase function in leucine catabolism and therefore that both the MCCase and GCCase enzymes might play important roles in the catabolic pathways for leucine as well as for acyclic terpenes.

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