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Biochemical and Structural Characterization of an Essential Acyl Coenzyme A Carboxylase from *Mycobacterium tuberculosis*

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Pathogenic mycobacteria contain a variety of unique fatty acids that have methyl branches at an even-numbered position at the carboxyl end and a long *n*-aliphatic chain. One such group of acids, called mycocerosic acids, is found uniquely in the cell wall of pathogenic mycobacteria, and their biosynthesis is essential for growth and pathogenesis. Therefore, the biosynthetic pathway of the unique precursor of such lipids, methylmalonyl coenzyme A (CoA), represents an attractive target for developing new antituberculous drugs. Heterologous protein expression and purification of the individual subunits allowed the successful reconstitution of an essential acyl-CoA carboxylase from *Mycobacterium tuberculosis*, whose main role appears to be the synthesis of methylmalonyl-CoA. The enzyme complex was reconstituted from the α biotinylated subunit AccA3, the carboxyltransferase β subunit AccD5, and the ε subunit AccE5 (Rv3281). The kinetic properties of this enzyme showed a clear substrate preference for propionyl-CoA compared with acetyl-CoA (specificity constant fivefold higher), indicating that the main physiological role of this enzyme complex is to generate methylmalonyl-CoA for the biosynthesis of branched-chain fatty acids. The α and β subunits are capable of forming a stable α6-β6 subcomplex but with very low specific activity. The addition of the ε subunit, which binds tightly to the α-β subcomplex, is essential for gaining maximal enzyme activity.

Mycobacterium tuberculosis remains one of mankind's deadliest pathogens, with approximately one-third of the world population infected and 2 million deaths each year (World Health Organization; www.who.int). Two features of the organism combine to make it one of the most serious disease-causing agents: its extreme infectivity and its ability to persist intracellularly to be reactivated later in life, a phenomenon that led to a deadly synergy with human immunodeficiency virus and AIDS (6, 16).

Anti-tuberculosis drug resistance is a major public health problem that threatens the success of the WHO-recommended treatment strategy for detection and cure of tuberculosis, as well as its global control. Whereas several antibiotics are effective in treating mycobacterial infections, these drugs target a surprisingly small number of essential functions in the cell (54). Therefore, the identification and characterization of the pathways that are required for mycobacterial growth would provide many new targets for the rational design of more effective antimycobacterial agents that could be active against drug-resistant strains (1).

Fatty acid biosynthesis is an emerging target for the development of novel antibacterial chemotherapeutics. The chemical mechanism in mammals is virtually identical to that of bacteria, but the protein sequences and arrangements of enzymatic active sites differ markedly between them so that specific inhibitors can be designed (11).

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The first committed step in the biosynthesis of long-chain fatty acids in all animals, plants, and bacteria is catalyzed by acetyl coenzyme A (CoA) carboxylase (ACC) (53). The reaction catalyzed by this enzyme involves two separate reactions:

$$HCO_3^- + \text{Mg-ATP} + \text{enzyme-biotin} \leftrightarrow \text{enzyme-biotin-}CO_2^- + \text{Mg-ADP} + P_i \quad (1)$$

$$\text{Enzyme-biotin-}CO_2^- + \text{acetyl-CoA} \leftrightarrow \text{enzyme-biotin} + \text{malonyl-CoA} \quad (2)$$

ACC is composed of three different components, which allow it to carry out these two distinct reactions. The biotin carboxylase component (BC) catalyzes the first half-reaction that involves the phosphorylation of bicarbonate by ATP to form a carboxyphosphate intermediate, followed by transfer of the carboxyl group to biotin to form carboxybiotin. In vivo, biotin is attached to the biotin carboxyl carrier protein (BCCP), designated above as enzyme-biotin, via an amide bond between the valeric acid side chain of biotin and the ε amino group of a specific lysine residue (18). In the second reaction, catalyzed by carboxyltransferase (CT), the carboxyl group is transferred from biotin to acetyl-CoA to form malonyl-CoA. Animals contain all three of these components on one polypeptide chain. In contrast, these three different components are on four separate polypeptides in the *Escherichia coli* form of ACC (37). Interestingly, several complexes with ACCase activity have been purified from a number of actinomycetes. Some of these complexes also possess the ability to carboxylate other substrates, including propionyl- and butyryl-CoA (22, 28, 30, 47). Consequently, these enzymes are referred to as acyl-CoA carboxylases (ACCase), and all of them consist

TABLE 1. Strains and plasmids used in this study

E. coli strain or plasmid	Description	Source or reference
Strains		
DH5α	F^- ϕ80*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 endA1 recA1 hsdR17 deoR supE44 thi-1 gyrA96 relA1*	27
BL21λ(DE3)	F^- *ompT* r_B^- m_B^- (DE3)	52
Rosetta λ(DE3)	pLacI^2 Cm^r; expresses rare tRNAs, facilitates expression of genes that contain rare *E. coli* codons	Novagen
Plasmids		
pGEM-T Easy	Used for cloning PCR products	Promega
pET22b(+)	Phagemid vector (Ap^r *lacZ′*) for expression of recombinant proteins under control of strong T7 transcription and translation signals	Novagen
pET24b(+)	Phagemid vector (Km^r *lacZ′*) for expression of recombinant proteins under control of strong T7 transcription and translation signals	Novagen
pET28a(+)	Phagemid vector (Km^r *lacZ′*) for expression of recombinant proteins under control of strong T7 transcription and translation signals	Novagen
pCY216	Vector containing *E. coli birA* gene	13
pD5	pET28a(+) with an insert carrying *accD5* His tag fusion gene under control of strong T7 transcription and translation signals	This work
p20D5	pET24a(+) with *accD5* under the control of strong T7 transcription and translation signals	This work
pRV3281	pET28a(+) with an insert carrying *Rv3281* His tag fusion gene under control of strong T7 transcription and translation signals	This work
pE5	pET28a(+) with an insert carrying *accE5* His tag fusion gene under control of strong T7 transcription and translation signals	This work
pA3	pET22a(+) with *accA3* under control of strong T7 transcription and translation signals	This work
pHA3	pET28a(+) with an insert carrying *accA3* His tag fusion gene under control of strong T7 transcription and translation signals	This work
pA2	pET22a(+) with *accA2* under control of strong T7 transcription and translation signals	This work
pA1	pET28a(+) with *accA1* under control of strong T7 transcription and translation signals	This work

of a large subunit (the α-chain) with the ability to carboxylate its covalently bound biotin group, and a smaller subunit (the β-chain) bearing the carboxyltransferase activity. In our laboratory, two ACCase complexes from *Streptomyces coelicolor* A3(2) have been widely characterized at both the biochemical and structural levels (19, 20, 46, 47). As a new feature of this group of enzymes, we found a third subunit, called ε, which is essential for the maximal activity of the complexes (20).

In addition to the usual fatty acids found in membrane lipids, mycobacteria have a wide variety of very-long-chain saturated (C_{18} to C_{32}) and monounsaturated (up to C_{26}) n-fatty acids as well as multimethyl-branched fatty acids present in several cell wall lipids that have been implicated in the ability of the organism to resist host defenses (10, 17, 43). Also, the occurrence of mycolic acids, long-chain high-molecular-weight α-alkyl β-hydroxy fatty acids, is a hallmark of mycobacteria and related species (34). Despite the essential role that fatty acids play in the formation of the lipids of the mycobacterial cell wall, little is known about the biochemistry and physiological role of the ACCases from *M. tuberculosis*. So far, the only carboxylase complex characterized from this organism had been isolated from crude extracts, and it showed, at least in vitro, higher affinity for propionyl-CoA compared with acetyl- or butyryl-CoA (45). In this paper, we present for the first time a genetic and biochemical characterization of an essential ACCase complex of *M. tuberculosis* and propose a physiological role for it based on the biochemical properties of the enzyme. These studies should be an important step forward towards the utilization of these new essential enzyme complexes as targets for the screening of new antimycobacterial drugs.

MATERIALS AND METHODS

Bacterial strains, culture, and transformation conditions. *E. coli* strain DH5α (27) was used for routine subcloning and was transformed according to Sambrook et al. (48). Transformants were selected on media supplemented with the appropriate antibiotics: 100 μg/ml ampicillin, 20 μg/ml chloramphenicol, or 50 μg/ml kanamycin. Strain BL21 λ(DE3) is an *E. coli* B strain lysogenized with λDE3, a prophage that expresses the T7 RNA polymerase from the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible *lacUV5* promoter (52). Rosetta λ(DE3) expresses rare tRNAs to facilitate expression of genes that encode rare *E. coli* codons. Strains, genotypes, and recombinant plasmids are listed in Table 1.

Growth conditions, protein production, and preparation of cell extracts. For the expression of heterologous proteins, *E. coli* strains harboring the appropriate plasmids were grown at 37°C in shake flasks in Luria-Bertani (LB) medium in the presence of the corresponding antibiotics for plasmid maintenance. To improve the biotinylation of AccA1-3 in *E. coli*, the strains containing pA1-3 were also transformed with pCY216 (13), which overexpresses the *E. coli* biotin ligase (BirA); 10 μM D-biotin was also added to the medium. Overnight cultures were diluted 1:100 in fresh medium and grown to an A_{600} of 0.5 to 0.8 before the addition of IPTG to a final concentration of 0.5 mM. Induction was allowed to proceed for 6 h at 20°C. The cells were harvested, washed, and resuspended in buffer A (50 mM Tris-HCl, pH 8, 300 mM NaCl, 0.75 mM dithiothreitol [DTT], 1 mM EDTA, 10% [vol/vol] glycerol).

Protein methods. Cell extracts and purified proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (36) using a Bio-Rad mini-gel apparatus. The final acrylamide monomer concentrations were 8, 10, or 18% (wt/vol) for the separating gel and 5% for the stacking gel. Coomassie brilliant blue was used to stain protein bands. Protein contents were determined by the method of Bradford (4) or Lowry (38) with bovine serum albumin as a standard. The biotinylated protein (AccA3) was detected by a modification of the Western blotting procedure described by Nikolau et al. (41). After electrophoretic separation, proteins were electroblotted onto nitrocellulose membranes (Bio-Rad) and probed with alkaline phosphatase (AP)-streptavidin conjugate (diluted 1:10,000) (Bio-Rad). Immunoblotting was performed according to Burnette (8) using anti-AccD5 in a 1:1,000 dilution. Anti-His (QIAGEN) was used at a 1:3,000 dilution. Antigenic polypeptides were visualized using an alkaline phosphatase-tagged secondary antibody. Antisera against AccD5 were elicited in rabbits following conventional procedures (8).

Coupled enzyme assay. The rate of ATP hydrolysis by biotin carboxylase was measured spectrophotometrically (32). The production of ADP was coupled to pyruvate kinase and lactate dehydrogenase, and the oxidation of NADH was monitored at 340 nm. Assays were performed in a microplate reader as previously described (20). Data were collected using a Dynex MRX microplate reader interfaced to a personal computer equipped with a data acquisition program. Initial velocities were obtained from initial slopes of the recorder traces. Under the assay conditions described, the reaction was linear for at least 3 min and the initial rate of reaction was proportional to the enzyme concentration. One unit of enzyme activity catalyzes the formation of 1 μmol of the respective carboxylated CoA derivative or ADP/min under the assay conditions described. Identical activities were measured by the $^{14}CO_2$ fixation method (5, 20, 30) and by the spectrophotometric method. Specific activity is expressed as units/mg of AccA3.

DNA manipulations. Isolation of plasmid DNA, restriction enzyme digestion, and agarose gel electrophoresis were carried out by conventional methods (48).

Gene cloning and plasmid construction. In all cases, the template for the PCRs was genomic DNA from *M. tuberculosis*.

pD5. The oligonucleotides AccD5up (5′-ATCGACTCATATGACAAGCGTTACCGAC), used to introduce an NdeI site (underlined) at the translational start codon of the *M. tuberculosis accD5* gene, and AccD5dwn (5′-TCACAAGCTTCGTTCGTTCCGCTCACTC), which introduces a HindIII site (underlined) at the end of the open reading frame (ORF), were used to amplify the complete *accD5*. To generate an *accD5* His tag fusion gene (full-length *accD5* fused to six His codons [His_6] at its N terminus), the PCR product was digested with NdeI and HindIII and cloned in NdeI-HindIII-cleaved pET28a, yielding pD5.

p20D5. To generate the expression of native AccD5 (without His tag), the NdeI-HindIII fragment from pD5 was cloned in NdeI-HindIII-cleaved pET24b (Novagen), yielding p20D5.

pA3. The oligonucleotides AccA3up (5′-ACAGGAGGCCATATGGCTAGTCACGC), used to introduce an NdeI site at the translational start codon of the *M. tuberculosis accA3* gene, and AccA3dwn (5′-GTTGAAGCTTCCGCCGGGCTTACTTG), which introduces a HindIII site at the end of the ORF, were used to amplify the complete *accA3*. The PCR product was digested with NdeI and HindIII and cloned in NdeI-HindIII-cleaved pET22a, yielding pA3.

pHA3. To generate an a*ccA3* His tag fusion gene (full-length *accA3* fused to six His codons at its N terminus), the plasmid pA3 was digested with NdeI and HindIII and cloned in NdeI-HindIII-cleaved pET28a, yielding pHA3.

pA2. The oligonucleotides AccA2up (5′-CGGATGTGATGCATATGGGAATCACTC), used to introduce an NdeI site at the translational start codon of the *M. tuberculosis accA2* gene, and AccA2dwn (5′-CGCTTTCAAGCTTGCTGGTGTCTGTCA), which introduces a HindIII site at the end of the ORF, were used to amplify the complete *accA2*. The PCR product was digested with NdeI and HindIII and cloned in NdeI-HindIII-cleaved pET22a, yielding pA2.

pA1. The oligonucleotides AccA1upNco (5′-CGGATGTGAGGCCATGGTTGACACC), used to introduce an NcoI site at the translational start codon of the *M. tuberculosis accA1* gene, and AccA1dwn (5′-GTTGTAAGCTTGATCCTAGTCCTTG), which introduces a HindIII site at the end of the ORF, were used to amplify the complete *accA1*. The PCR product was digested with NcoI and HindIII and cloned in NcoI-HindIII-cleaved pET28a, yielding pA1.

pRv3281. The oligonucleotides E5UP (5′-AACATATGGGAACGTGCCCCTGTGAGTC), used to introduce an NdeI site at the translational start codon of the *M. tuberculosis* ORF Rv3281, and E5bDWN (5′-CAGGGAAGCTTGACCCGAGCACCAG), which introduces a HindIII site at the end of the ORF, were used to amplify the complete ORF Rv3281. To generate a His tag fusion gene (ORF Rv3281 product fused to six His codons at its N terminus), the PCR product was digested with NdeI and HindIII and cloned in NdeI-HindIII-cleaved pET28a, yielding pRv3281.

pE5. The oligonucleotides E5bUP (5′-GGCCCATATGACCGAAGAAGCCGCTG), used to introduce an NdeI site at Val_{102} of ORF Rv3281, and E5bDWN (5′-CAGGGAAGCTTGACCCGAGCACCAG), which introduces a HindIII site at the end of the ORF, were used to amplify the partial ORF Rv3281 (called AccE5). To generate an *accE5* His tag fusion gene (*accE5* fused to six His codons at its N terminus), the PCR product was digested with NdeI and HindIII and cloned in NdeI-HindIII-cleaved pET28a, yielding pE5.

Protein purification protocols. For expression of tagged proteins, the plasmids pD5 (His_6-AccD5), pRv3281, and pE5 (His_6-AccE5) were transformed into *E. coli* BL21(DE3) or Rosetta(DE3) $pLacI^2$. Protein expression was induced by adding 0.5 mM IPTG to cultures grown to an A_{600} of 0.8. Cells were resuspended in buffer A and disrupted by sonication, and the lysate was clarified by centrifugation at 20,000 × *g* and 4°C for 30 min. The supernatant was applied to a Ni^{2+}-nitrilotriacetic acid (NTA)-agarose affinity column (QIAGEN), equilibrated with the same buffer supplemented with 20 mM of imidazole. The column was subsequently washed, and the His_6-tagged proteins were eluted from the column using binding buffer containing 60 to 250 mM imidazole. Fractions of the eluate were collected and analyzed for protein by SDS-PAGE. The fractions containing purified proteins were dialyzed at 4°C overnight against 100 mM potassium phosphate, pH 7.6, 0.75 mM DTT, 1 mM EDTA, and 20% glycerol (vol/vol). Proteins were stored at −80°C.

The α subunits (AccA1 to -3) were purified from cultures of BL21(DE3) harboring pA1-3 and pCY216, which provides the *E. coli birA* gene to allow a high level of biotinylation of the biotin-containing proteins. Protein expression was induced by addition of 0.5 mM IPTG and 0.5% arabinose (induction of biotin ligase). Cells were pelleted, resuspended in buffer A, and disrupted by sonication. The lysate was clarified by centrifugation at 20,000 × *g* at 4°C for 30 min. DNase was added to degrade nucleic acid. Ammonium sulfate was added to 50%, and the supernatant was removed by centrifugation at 20,000 × *g* at 4°C for 30 min. The protein precipitate was resuspended in buffer A and dialyzed overnight against the same buffer. The dialyzed solution was applied to a column of avidin-Sepharose (Promega), equilibrated with the same buffer. The column was subsequently washed with 10 volumes of binding buffer; biotinylated proteins were eluted from the column using binding buffer containing 5 mM biotin. The fractions containing purified protein were dialyzed at 4°C overnight against 100 mM potassium phosphate, pH 7.6, 0.75 mM DTT, 1 mM EDTA, and 20% (vol/vol) glycerol.

Interaction assay. The interaction of AccD5 with AccA3 and AccE5 was studied in vitro by mixing 200 μg of cell extracts expressing AccD5, AccA3, and His_6-AccE5 from p20D5, pA3, and pE5, respectively. The interaction of AccD5 with AccA3 was studied by mixing 200 μg of cell extracts expressing AccD5 and His_6-AccA3 from p20D5 and pHA3, respectively. After incubation for 1 h at 4°C, the mixture was passed through an Ni^{2+}-NTA agarose affinity column, equilibrated and washed (10 volumes) with buffer A, and eluted with the same buffer containing 40 to 250 mM imidazole. The outcome of this column was evaluated by SDS-PAGE and Western blotting using different antibodies: anti-His for the detection of His_6-AccE5, anti-AccD5 for AccD5, and alkaline phosphatase-streptavidin conjugate (Bio-Rad) for AccA3.

Stoichiometry of the α-β subcomplex. Molecular mass was estimated by size exclusion chromatography using an ΔKTA basic high-performance liquid chromatograph (Amersham). Samples containing 200 μg of AccA3, AccD5, or a mixture of both proteins were loaded onto a Superdex S200 column (Amersham) equilibrated in 50 mM potassium phosphate, pH 7.6, 50 mM NaCl, and 0.5 mM DTT and eluted with the same buffer. The column was calibrated with the following molecular mass standards: blue dextran, 200,0000; thyroglobulin, 669,000; apoferritin, 443,000; β-amylase, 200,000; alcohol dehydrogenase, 150,000; bovine serum albumin, 66,000; and lysozyme, 14,300. All fractions were analyzed by SDS-PAGE.

RESULTS

Predictive analysis of the putative ACCase of *M. tuberculosis*. From the analysis of the *M. tuberculosis* genome, one can predict that there are three genes coding for ACCase-related α subunits (*accA1* to *-3*) and six for the β subunits (a*ccD1* to *-6*). There is also an ORF (Rv3281) associated with the β subunit *accD5*; this small gene codes for a putative ε subunit with high homology to the ε subunits characterized in *S. coelicolor* (20). It has been postulated that two of the putative carboxylase complexes (AccA1-AccD1 and AccA2-AccD2) are probably involved in the degradation of odd-numbered fatty acids, as they are adjacent to genes encoding putative fatty acid degradative enzymes (14). However, neither the function nor the subunit composition of any of these complexes has been previously described. Interestingly, from a recent Himar1-based transposon mutagenesis, ORF Rv3281 and *accA3*, *accD4*, and *accD6* were found to be essential genes (49).

To characterize these complexes, we performed phylogenetic analysis, including the information for most of the predicted β subunits (CT) associated with the carboxylase group of enzymes from actinomycetes (2, 7, 12, 14, 15, 25, 31, 33, 42). The primary structure of 38 polypeptides was analyzed using Clustal W (version 1.7) and refined by visual inspection. The

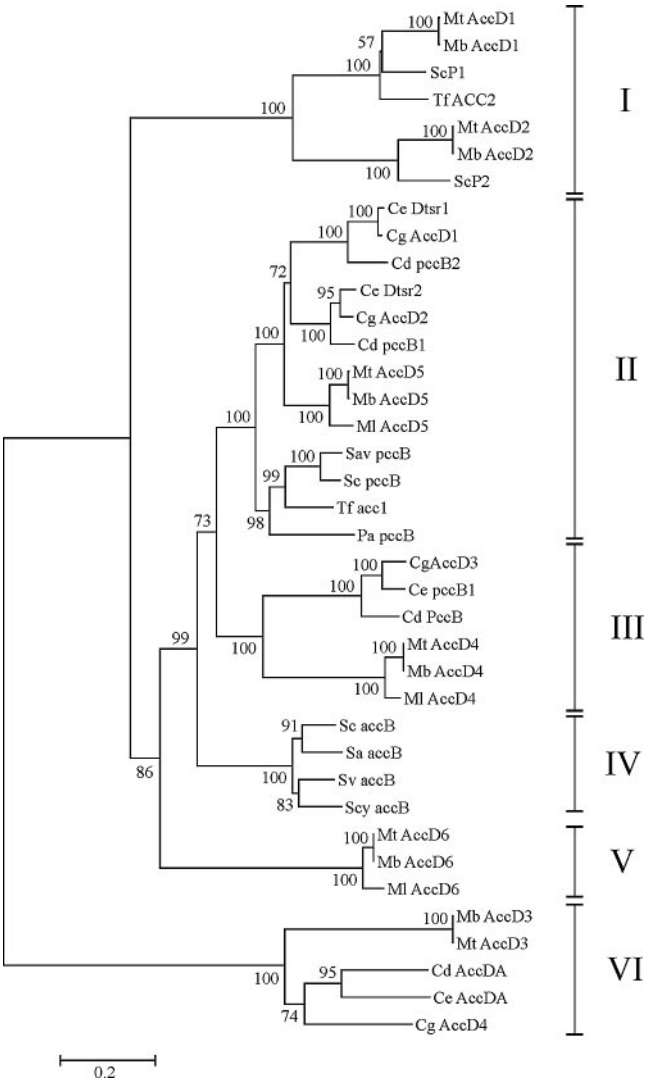

FIG. 1. Phylogenetic analysis of β subunits of ACCase complexes found in actinomycetes. The consensus phylogenetic tree was constructed by Bayesian inference. All the bootstrap values are indicated. The lengths of the branches are proportional to the inferred evolutionary distances. The scale (number of substitutions per site) is shown at the bottom. The organism key is as follows: Mt, *Mycobacterium tuberculosis*; Mb, *Mycobacterium bovis*; Ml, *Mycobacterium leprae*; Ce, *Corynebacterium efficiens*; Cd, *Corynebacterium diphtheriae*; Cg, *Corynebacterium glutamicum*; Sa, *Streptomyces antibioticus*; Sav, *Streptomyces avermitilis*; Sc, *Streptomyces coelicolor*; Scy, *Streptomyces cyanogenus*; Sv, *Streptomyces venezuelae*; Tf, *Thermobifida fusca*; Pa, *Propionibacterium acnes*.

tree was constructed by Bayesian inference (35), and the results showed several remarkable features (Fig. 1). Clearly, several defined groups can be distinguished (I to VI). Group I includes the two CTs corresponding to the *Mycobacterium* carboxylase complexes that have been implicated in the degradative metabolism of odd-numbered fatty acids, as mentioned previously (14). Group II is the only one where each organism analyzed is represented at least once, suggesting that members of this group might be involved in fundamental core functions in actinobacteria. *S. coelicolor* PccB is the only member of this group that has been characterized in detail. This carboxyltransferase is able to recognize propionyl- and butyryl-CoA as substrates (20, 47) but not acetyl-CoA, suggesting that members of this group are probably the β subunits of propionyl-CoA carboxylase (PCC) complexes.

In contrast, we can only find members of the mycobacterial branch of actinomycetes in group III, suggesting that they represent CTs with more specialized functions. In agreement with this finding, it has been recently demonstrated that members of this group are directly involved in mycolic acid biosynthesis (24, 44). No mycobacterial proteins are present in group IV. This is particularly interesting as members of this group are probably essential ACCase complexes that primarily recognize acetyl-CoA as a substrate, as has been demonstrated for the AccB subunit of *S. coelicolor* (20, 46). In group V, we can only find proteins corresponding to the *Mycobacterium* genus. We have described previously that the presence of an isoleucine residue in position 420 of the essential CT subunit of *S. coelicolor*, AccB, is directly related to the ability of this subunit to recognize acetyl-CoA as a substrate (19). Interestingly, members of group V also have an isoleucine in this position, suggesting that this group of proteins could be essential ACCs that have diverged from other known ACCs due to the intracellular lifestyle of this group of bacteria. Moreover, AccD6 of *M. tuberculosis* was shown to be an essential protein for the viability of this microorganism. Recently it has been suggested that members of the most distantly related group, VI, are not directly involved in lipid biosynthesis (24), and it is hard to predict their function since none of the other members of this group have a known activity or a predicted function. Interestingly, all of the members of the individual groups share the same amino acid in the position equivalent to position 420 of AccB, suggesting that they might have very similar substrate specificity.

Another interesting feature that arises from our sequence alignment analysis is that all of the β subunits present in groups II and IV have a putative ε subunit genetically associated with the corresponding β-subunit-encoding genes, suggesting that the new group of ACCases described previously only for streptomycetes is ubiquitously present in all the actinomycetes analyzed so far.

As stated above, the ORF coding for the putative ε subunit in *M. tuberculosis* was found to be essential (49). This new class of ACCases (containing α, β, and ε subunits) could be considered as specific targets for the development of new antimycobacterial drugs based on their distinctive structure. Thus, we set out to characterize the putative essential carboxylase complex containing AccD5 as the β subunit at the biochemical and structural levels.

Identification of the subunits of the ACCase 5 complex. Considering that the genetic organization of the ACCase 5 complex resembles that of the PCC characterized from *S. coelicolor* (2, 47), one could predict that AccA3 is the α subunit of the ACCase 5 complex from *M. tuberculosis* (Fig. 2A). To confirm this hypothesis, we expressed and purified from *E. coli* the three possible components of the complex, AccA3, the β subunit AccD5, and the putative ε subunit Rv3281, the last two proteins containing His tags at the N terminus.

It was shown previously that ACCase complexes from some actinomycetes have a 1:1 molar ratio between the α and β subunits (20, 26). Therefore, we assayed the activity of AccD5

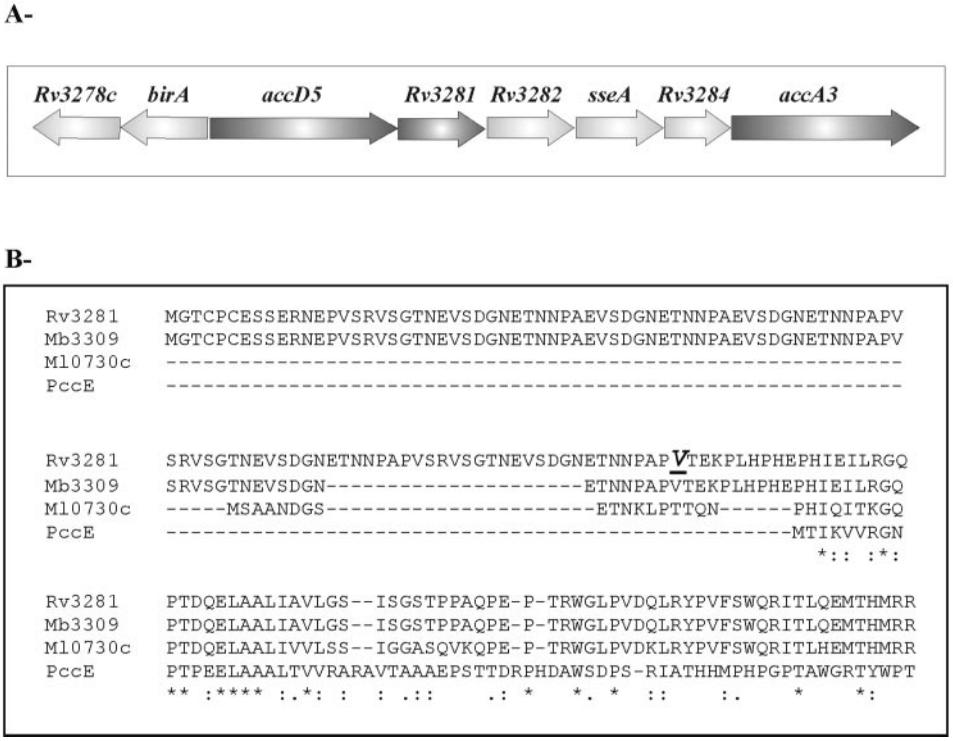

FIG. 2. (A) Genetic organization of ACCase 5 complex. (B) Amino acid sequence alignment of putative ε subunits from mycobacteria and PccE from *S. coelicolor*. Amino acid sequences of the ε subunit of *S. coelicolor* (PccE) and the putative subunits from *M. tuberculosis* (Rv3281), *M. leprae* (Ml0730c), and *M. bovis* (Mb3309) are shown. Identical amino acids are indicated by asterisks below, and conserved residues are indicated by single dots or stacked dots. The V_{102} start codon of AccE5 is underlined.

in the presence of AccA3 in a 1:1 molar ratio plus the addition of 10 molar excess of the product of Rv3281, in analogy with the conditions used for the ACC and PCC complexes of *S. coelicolor* (20). We only found low levels of activity using saturating concentrations (1 mM) of either acetyl- or propionyl-CoA as a substrate (Table 2). Interestingly, the presence of Rv3281 showed no effect on the activity of the AccD5 complex, since we obtained similar enzyme activity levels in the absence of this protein.

Analysis of the gene encoding the putative ε subunit (Rv3281) revealed that the first 308 bp contain a stretch of several direct repeats. The fact that some of these repeats were absent in the homologous gene of *Mycobacterium leprae* and *Mycobacterium bovis* suggested that the functional protein was perhaps shorter than the predicted complete ORF; therefore, we cloned and expressed a smaller protein starting in Val_{102} of the Rv3281 ORF, which we named AccE5 (Fig. 2B). When AccE5 was added in a 10 M excess with respect to the α-β subunits, a clear stimulatory effect on the enzyme activity was observed (Table 2).

TABLE 2. In vitro reconstitution of the ACCase 5 complex

Proteins	Activity (mU min^{-1} mg $AccA3^{-1}$)[a]	
	Propionyl-CoA (1 mM)	Acetyl-CoA (1 mM)
AccA3 + AccD5	50 ± 6	21 ± 7
AccA3 + AccD5 + Rv3281	48 ± 5	20 ± 9
AccA3 + AccD5 + AccE5	612 ± 25	98 ± 15

[a] Enzyme activity was measured by the coupled assay method.

The activity of the ACCase 5 complex depends on the concentration of AccE5. Titration of the effects of AccE5 on the ACCase 5 complex activity was performed in reaction mixtures containing equimolar amounts (0.4 μM) of AccA3-AccD5 and saturating concentrations of the substrate (1 mM propionyl-CoA). Within the limits of the assay, very low activity could be measured in the absence of AccE5 and the rate of product formation depended on the AccE5 concentration (Fig. 3). The data fit to a sigmoidal curve, implying that a threshold concentration of epsilon is needed before it can exert its biological function. Saturation (more than 90% of maximal velocity) was achieved at molar ratios higher than 5:1 for AccE5, with respect to either the α or β subunit.

Kinetic parameters of the ACCase 5 complex. The kinetic characterization of the ACCase 5 complex was performed at a 1:1:10 molar ratio between the α, β, and ε subunits, respectively, as this concentration was deemed optimal with respect to α and β and saturating with respect to ε (see above). The reconstituted enzyme was kinetically characterized in terms of K_m, V_{max}, and substrate specificity. Table 3 shows the values obtained for each parameter with two different acyl-CoA derivatives, acetyl- and propionyl-CoA. The affinities of the ACCase 5 complex for both of these substrates were approximately the same; however, the enzyme was significantly more active with propionyl-CoA as the V_{max} for this compound was almost fivefold higher than that for acetyl-CoA. In all cases,

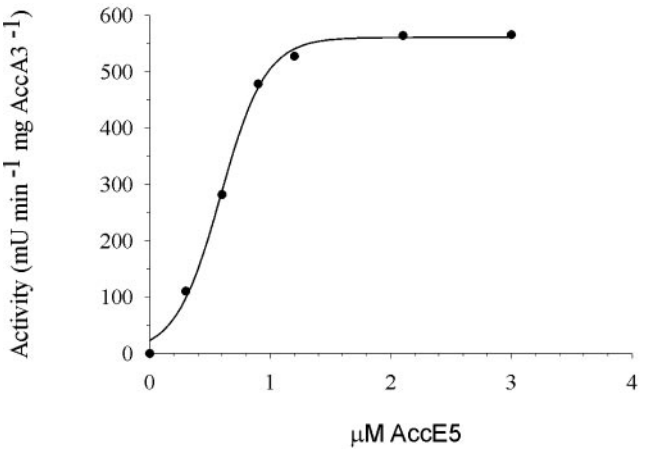

FIG. 3. Effects of AccE5 on the ACCase 5 complex activity. Increasing concentrations of the AccE5 subunit were added to the assay media containing equimolar amounts (0.4 μM) of AccA3 and AccD5 subunits.

the kinetics were hyperbolic. C_4 to C_{20} acyl-CoA esters were also tested as possible substrates for this enzyme complex, but no activity was detected with any of these compounds (not shown).

Analysis of protein-protein interaction in the ACCase 5 complex. The interaction of AccD5 with AccA3 and AccE5 was studied in vitro by mixing cell extracts expressing AccD5, AccA3, or His_6-AccE5 from p20D5, pA3, and pE5, respectively. After incubation for 1 h at 4°C, the mixture was passed through an Ni^{2+}-NTA agarose affinity column, equilibrated and washed (10 volumes) with buffer A, and eluted with the same buffer containing increasing concentrations of imidazole (40 to 250 mM). The outcome of this column was evaluated by SDS-PAGE, and the result is shown in Fig. 4A. Two protein bands with molecular masses of approximately 67 and 57 kDa (corresponding to AccA3 and AccD5) were present in the fractions corresponding to the washes of the column, probably due to the high concentration of these proteins in the crude extracts. Interestingly, these two bands significantly increased their concentration in those fractions where His_6-AccE5 started to elute from the affinity column (elution fractions 2 to 5), indicating the existence of strong protein-protein interactions between these subunits. The two protein bands that coeluted with His_6-AccE5 were confirmed to be AccA3 and AccD5 by immunoblotting experiments (Fig. 4B and C). Moreover, we could not detect any nonspecific interaction of AccA3 or AccD5 with the Ni^{2+}-NTA column in control experiments run in the absence of the ε subunit (Fig. 4D), confirming that the coelution of the three subunits seen in Fig. 4A was due to a ready interaction of the α and β subunits with ε.

We also studied the interaction of the α and β subunits in the absence of ε. Cell extracts containing His_6-AccA3 and AccD5 were mixed and passed them through a Ni^{2+}-NTA agarose affinity column. Interestingly, we found that these two subunits do interact in the absence of ε (Fig. 5). This is in clear contrast with the results found for the *S. coelicolor* ACC complex where AccE was essential for the interaction between AccB and AccA2 (20). Experiments directed to determine whether ε binds to the α or β subunits independently gave negative results (data not shown), confirming that AccE5 binds exclusively to the α-β subcomplex and not to the individual subunits.

To determine the oligomeric state of the α and β subunits and the stoichiometry of the α-β subcomplex, we performed size exclusion chromatography of the individual subunits and of the protein mix. Figure 6A shows that AccD5 runs as an hexamer while the oligomeric state of AccA3 is a mix of hexamers and trimers. When both proteins were preincubated and then run through a Superdex S200 column, a new peak corresponding to a dodecamer, with the molecular mass of AccA3 plus AccD5, was observed. To determine the subunit composition of the new complex, SDS-PAGE was run with the different fractions eluted from the column. As shown in Fig. 6B, the fraction corresponding to the dodecamer contained both proteins, AccA3 and AccD5, in a 1:1 molar ratio. These results are in agreement with the reported α6/β6 structure of an acyl-CoA carboxylase previously purified from *M. smegmatis* (26, 28).

DISCUSSION

Despite efforts at understanding the biosynthesis of lipids from *M. tuberculosis*, little is known about the enzymes that catalyze the formation of the building blocks of these complex molecules. In this study, the successful in vitro reconstitution of an ACCase complex from its purified components allowed us to carry out, for the first time, a primary characterization of an essential carboxylase of this microorganism at a biochemical and genetic level. This complex is formed by the biotinylated α subunit AccA3, the carboxyltransferase β subunit AccD5, and the small ε subunit coded for by Rv3281, which we named AccE5. Our results show that AccE5 is necessary for the ACCase complex to reach maximal enzyme activity. Interestingly, the physical interaction between AccE5 and the other subunits differed from what was observed for the ACC and PCC complexes of *S. coelicolor* (20). In these two cases, the ε subunit interacted with the specific CT component establishing a β-ε subcomplex, which only then was able to interact with the α subunit. Thus, we predicted that ε function was to bring together the two catalytic subunits α and β. For the ACCase 5 complex, the α and β subunits are able to form a stable α-β subcomplex in the absence of ε (Fig. 5); however, the catalytic activity of this complex is barely detectable under the assay conditions used. The presence of ε at a 5:1 ratio with respect to the α or the β subunits stimulated the catalytic activity at least 20-fold (Fig. 3). This result suggests that an enzyme conformational change is induced in such a way that the active site might become accessible to the substrate, when ε binds to the α-β subcomplex.

TABLE 3. Kinetic parameters of the ACCase 5 complex

Parameter[a]	Value for substrate	
	Propionyl-CoA	Acetyl-CoA
K_m (μM)	240 ± 35	220 ± 55
V_{max} (mU min^{-1} mg $AccA3^{-1}$)	680 ± 43	120 ± 8
Specificity constant	2.8	0.5

[a] Enzyme activity was measured by the coupled assay method. The specificity constant is calculated as V_{max}/K_m. The activity for C_4 to C_{20} acyl-CoAs was lower than 3 mU min^{-1} mg $AccA3^{-1}$.

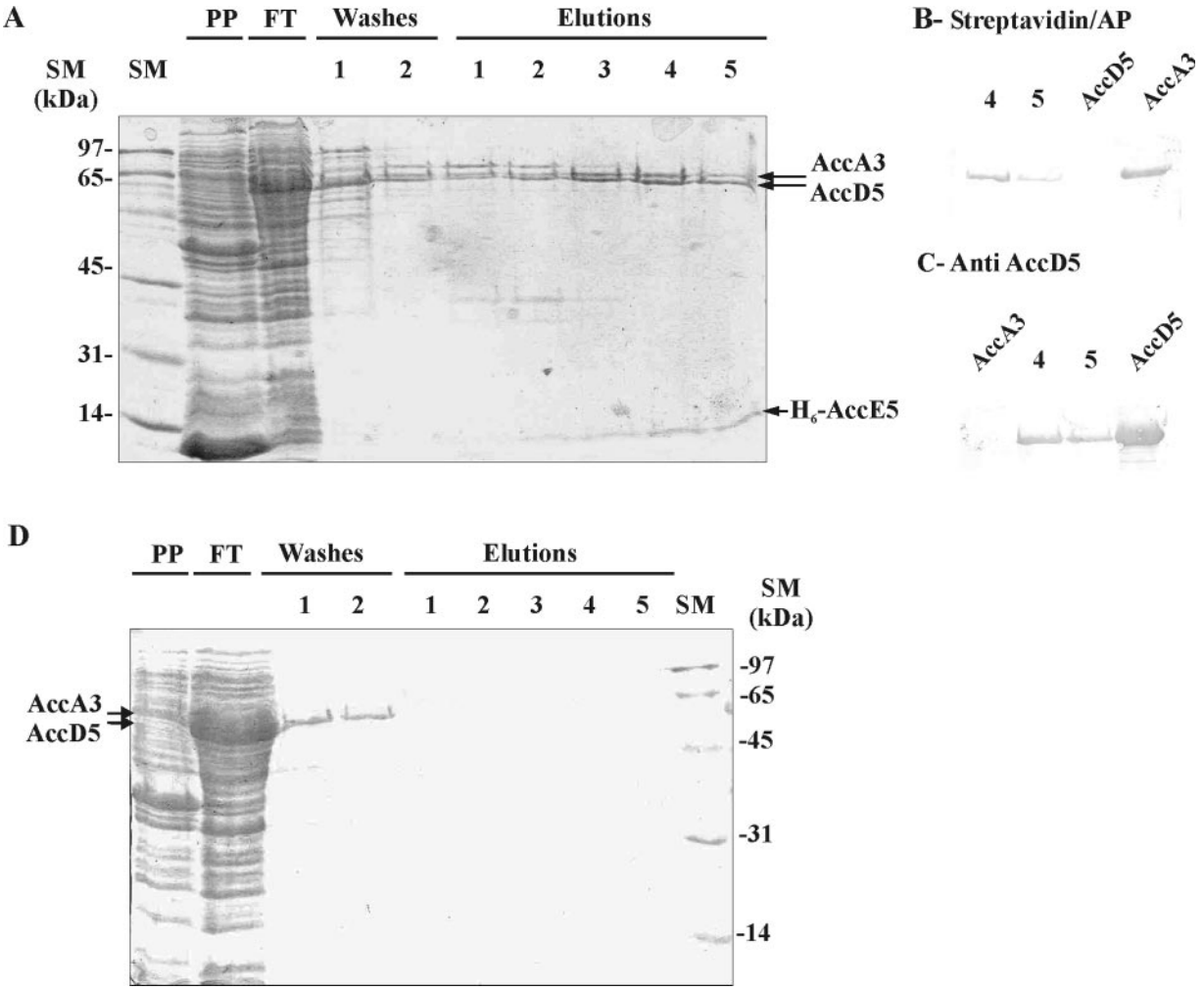

FIG. 4. Evidence of AccA3-AccD5-AccE5 interaction by copurification from an Ni^{2+} affinity column. A mixture of cell extracts expressing AccD5, AccA3, or His_6-AccE5 was loaded on an Ni^{2+} affinity column, washed with buffer A, and eluted with the same buffer containing 40, 60, 80, 100, and 250 mM imidazole (elution fractions 1 to 5). Twenty microliters of each fraction was run on an 18% SDS-PAGE gel and Coomassie blue stained. SM, standard molecular mass markers; PP, pellet; FT, flowthrough (A). Duplicates of some fractions were run on SDS-PAGE, transferred to nitrocellulose, and probed with alkaline phosphatase-streptavidin conjugate (B) or anti-AccD5 antibodies (C). The same experiment was done in the absence of His_6-AccE5, and 20 μl of each fraction was run on 18% SDS-PAGE and Coomassie blue stained (D).

Alternatively, the ε subunit could change the oligomeric composition of the α-β subcomplex to make it functional. The crystal structure of the different subunits and of the whole complex will be necessary to understand the real function of ε in this class of enzymes.

We have previously shown that the presence of an ε subunit in the ACCase complexes is widely distributed in *Streptomyces* (20). Here, we found that this subunit is also present in all the actinomycetes analyzed, suggesting that this component was present in the common ancestor of this group of microorganisms. Interestingly, this ORF was found to be essential for *M. tuberculosis* viability (49), and it was included with the 219 essential core genes that all *Mycobacteria* have conserved during evolution (39). Particularly for *M. leprae*, which suffered extensive reductive evolution, the conservation of this gene strongly suggests that it encodes a protein of essential function (39).

Recently, it was suggested that an ACCase complex containing two different β subunits, AccD4 and AccD5, is involved in the carboxylation of the fatty acid that will be found as the α chain of the mycolic acids prior to the final condensation step by Pks13 to produce mycolic acid precursors in *Mycobacterium smegmatis* (44). Interestingly, we showed that for *M. tuberculosis*, an active ACCase complex with kinetic parameters comparable to those found in other systems, can be fully reconstituted with the ortholog of only one of these two β subunits, AccD5. Our attempts to demonstrate the formation of AccD4-AccD5 hetero-oligomers by mixing Flag-AccD4 and His_6-AccD5 of *M. tuberculosis* were unsuccessful (data not shown), suggesting that the results observed by Portevin et al. (44) cannot be explained simply by the formation of hetero-oligomers of different β subunits, as could be predicted based on the hexameric composition of these CTs (19). One explanation for these results would be that macro complexes formed by the interaction of the individual AccD4-AccA3 and AccD5-AccA3 complexes could occur within the cell or during the processing of the cell extracts, accounting for the coimmunoprecipitation of

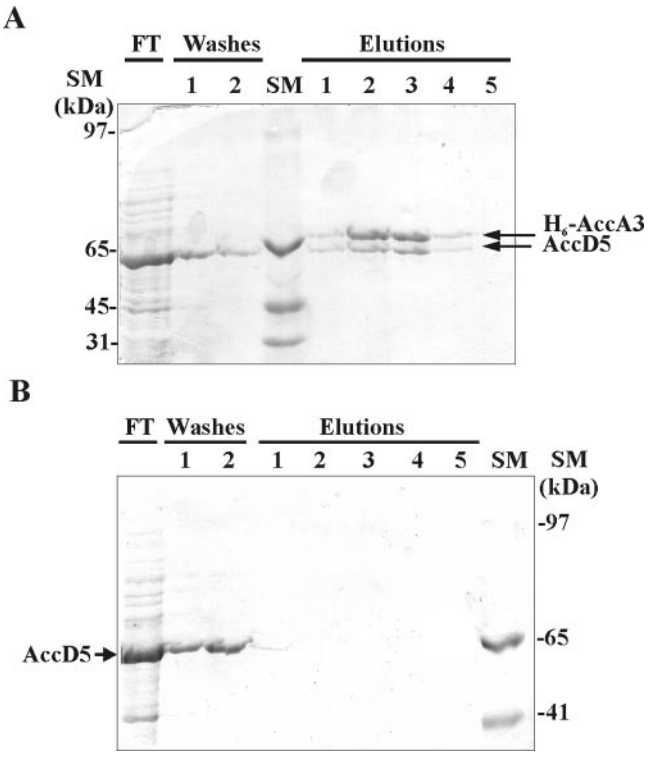

FIG. 5. Interaction of the α and β subunits in the absence of ε. A mixture of cell extracts containing either AccD5 and His_6-AccA3 (A) or AccD5 (B) was loaded on an Ni^{2+} affinity column, washed with buffer A, and eluted with the same buffer containing 40, 60, 80, 100, and 250 mM imidazole (elution fractions 1 to 5). Twenty microliters of the eluted fractions was run on 10% SDS-PAGE and Coomassie blue stained. SM, standard molecular mass markers; FT, flowthrough.

the two β subunits (44). The possibility of reconstituting in vitro the ACCase complexes from their pure components, as shown in this study, now opens the way to determine unambiguously the subunit composition and the substrate specificity of each of these enzyme complexes.

The kinetic characterization of the ACCase 5 showed unique substrate specificity characteristics. Although this complex is able to carboxylate acetyl-CoA, its preferred substrate is propionyl-CoA (Table 2). In a recent paper, we demonstrated that the amino acid residue present at position 420 of the β subunits corresponding to the ACC and PCC complexes of *S. coelicolor* plays a key role in determining the substrate specificity of these enzymes (19). More specifically, we found that the presence of an aspartic residue at position 422 is related to the ability of PccB to carboxylate propionyl-CoA and an isoleucine in position 420 confers to AccB the ability to carboxylate both acetyl- and propionyl-CoA (19). We also constructed several mutant proteins and found that the replacement of the aspartic residue of PccB by a cysteine does not alter the specificity for propionyl-CoA but confers the ability to carboxylate acetyl-CoA, although at lower levels (Arabolaza et al., unpublished results). This is in agreement with the results presented in this work as the β subunit AccD5 has a cysteine in this position.

The carboxylation of propionyl-CoA by ACCase 5 is one of the two putative metabolic pathways that *M. tuberculosis* could use to synthesize the methylmalonyl-CoA necessary for the synthesis of the complex lipids found in this pathogen. Alternatively, the isomerization of succinyl-CoA could be accomplished by the methylmalonyl-CoA mutase (3, 29). However, although there are genes coding for the two putative subunits of the methylmalonyl-CoA mutase in *M. tuberculosis* (14), nothing is known about the physiological relevance of this route except that it is not essential for *M. tuberculosis* viability

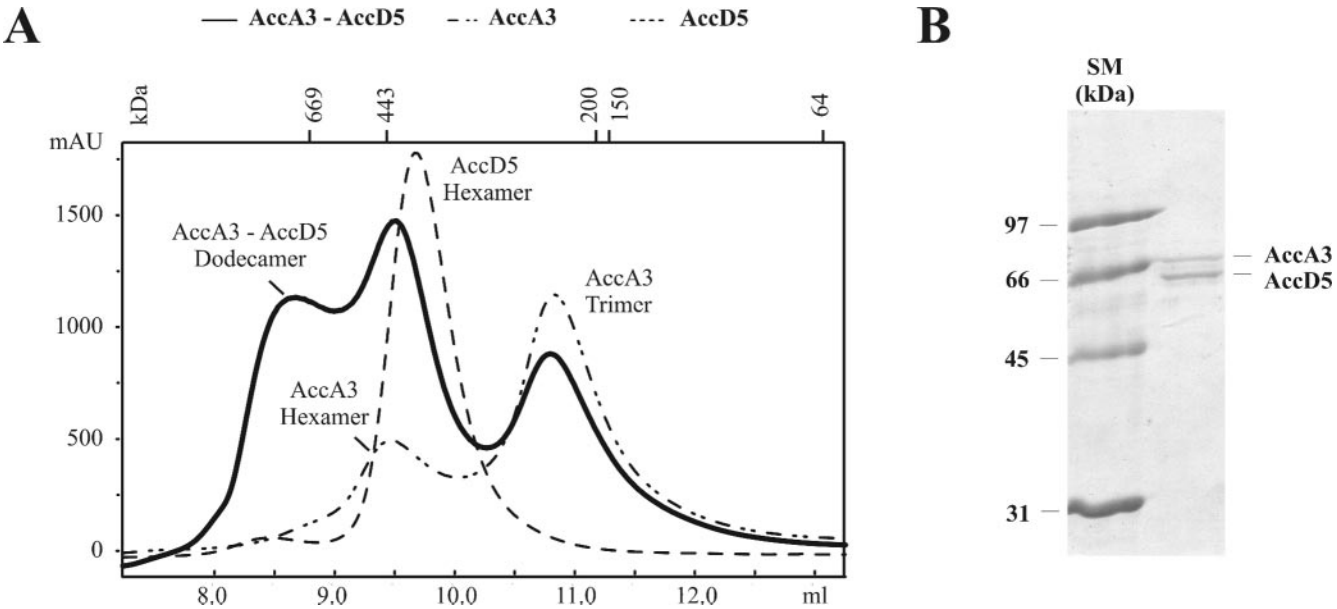

FIG. 6. Determination of the stoichiometry of the α-β subcomplex. AccA3, AccD5, or a mixture of both proteins was analyzed by size exclusion chromatography. The protein profiles (A_{215} in milli-absorbance units [mAU]) and molecular masses of protein standards used for calibration are shown (A). The fraction corresponding to the dodecamer was analyzed by SDS-PAGE (B). SM, standard molecular mass markers.

(49). Methylmalonyl-CoA is used as an extender unit by several enzymes for the synthesis of most of the complex lipids in *M. tuberculosis*, including some polyketide synthases (24, 51), mycoceric acid synthases (*mas*) (23, 40), and *mas*-like proteins (21). Some of the lipids synthesized by these enzymes are surface exposed, such as phthiocerol esters and mycosides that have been found to be unique to pathogenic mycobacteria and play important functions in mycobacterial interaction with the host (17, 50). In addition to being important virulence factors, these *M. tuberculosis* extractable lipids also play a role in cell envelope architecture and permeability (9). Thus, methylmalonyl-CoA biosynthesis by ACCase 5 is probably the only pathway utilized by *M. tuberculosis* for generating the substrates for the biosynthesis of some essential fatty acids. Finally, we cannot rule out that ACCase 5 could also function as an ACC enzyme to generate malonyl-CoA for fatty acid biosynthesis, although we showed that this complex is at least five times more efficient as a PCC.

The proposed essentiality of ACCase 5 together with its unique structural properties makes this enzyme complex a very interesting target for the development of a highly specific antimycobacterial drug. The successful in vitro reconstitution of the ACCase 5 complex presented in this study complemented with the determination of the crystal structure of the β subunit (work in progress) will provide the tools for structure-based design and high-throughput enzyme inhibition assays involved in the identification of new lead compounds against *M. tuberculosis*.

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