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AccD6, a Member of the Fas II Locus, Is a Functional Carboxyltransferase Subunit of the Acyl-Coenzyme A Carboxylase in Mycobacterium tuberculosis

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The Mycobacterium tuberculosis acyl-coenzyme A (CoA) carboxylases provide the building blocks for de novo fatty acid biosynthesis by fatty acid synthase I (FAS I) and for the elongation of FAS I end products by the FAS II complex to produce meromycolic acids. The M. tuberculosis genome contains three biotin carboxylase subunits (AccA1 to -3) and six carboxyltransferase subunits (AccD1 to -6), with accD6 located in a genetic locus that contains members of the FAS II complex. We found by quantitative real-time PCR analysis that the transcripts of accA3, accD4, accD5, and accD6 are expressed at high levels during the exponential growth phases of M. tuberculosis in vitro. Microarray analysis of M. tuberculosis transcripts indicated that the transcripts for accA3, accD4, accD5, accD6, and accE were repressed during later growth stages. AccD4 and AccD5 have been previously studied, but there are no reports on the function of AccD6. We expressed AccA3 (α3) and AccD6 (β6) in E. coli and purified them by affinity chromatography. We report here that reconstitution of the α3-β6 complex yielded an active acyl-CoA carboxylase. Kinetic characterization of this carboxylase showed that it preferentially carboxylated acetyl-CoA (1.1 nmol/mg/min) over propionyl-CoA (0.36 nmol/mg/min). The activity of the α3-β6 complex was inhibited by the ε subunit. The α3-β6 carboxylase was inhibited significantly by dimethyl itaconate, C75, haloxyfop, cerulenin, and 1,2-cyclohexanedione. Our results suggest that the βε subunit could play an important role in mycolic acid biosynthesis by providing malonyl-CoA to the FAS II complex.

Tuberculosis causes 2 million deaths each year, according to the World Health Organization. Mycobacterium tuberculosis, the pathogen that causes the disease, infects 8 million people each year and is one of the world’s deadliest pathogens (9). The ongoing AIDS pandemic has developed a deadly synergy with tuberculosis, which is the leading cause of death among AIDS patients (2). Multidrug-resistant M. tuberculosis strains have been emerging rapidly (9), and the need for identifying novel drug targets in this pathogen has become urgent. The cell wall of M. tuberculosis is lipid enriched and acts as an impermeable barrier to many common broad-spectrum antibiotics (14).

The first committed step of fatty-acid biosynthesis, which is the biotin-dependent carboxylation of acyl-coenzyme A (CoA) to produce malonyl-CoA and methylmalonyl-CoA, is catalyzed by the acyl-CoA carboxylase. The reaction consists of two catalytic steps, which involve the biotin carboxylase and the carboxyltransferase (8). In M. tuberculosis, the biotin carboxylation step is catalyzed by the α subunit; there are three open reading frames (ORFs) that can encode the α subunit (accA1 to -A3) in the genome. Carboxyl transfer is catalyzed by the β subunit, and there are six β subunits (accD1 to -D6) in the genome of the pathogen (6).

Previously, the catalytic activities of the α5, β4, and β5 subunits were studied (10, 11, 22, 24). However, the levels of expression of the various subunits have not been examined. Our analysis of transcripts from M. tuberculosis cells indicate that the α3, β4, β5, and ε ORFs are the main subunits regulated during cell growth. To determine whether the highly expressed βε subunit possessed enzymatic activity and to assess its substrate specificity, we expressed and purified the βε subunit and reconstituted it with the purified α3 subunit. We report that an active acyl-CoA carboxylase was reconstituted with the purified βε and α3 subunits and that it preferentially carboxylated acetyl-CoA over propionyl-CoA. This is the first report showing that βε, which is a member of a fatty acid synthase II (FAS II) gene locus, is a functional carboxyltransferase of the acyl-CoA carboxylase in M. tuberculosis, and these results suggest that βε might make a significant contribution to mycolate biosynthesis.

MATERIALS AND METHODS

Bacterial strains and culture conditions. M. tuberculosis H37Rv was grown in Middlebrook 7H9 (supplemented with 0.05% Tween 80, 10% oleic acid-albumin-dextrose-catalase enrichment, and 0.2% glycerol). All subcloning procedures were carried out in Escherichia coli DH5α according to the method of Sambrook et al. Recombinant-protein expression was performed in E. coli BL21 Star (DE3) (In Vitrogen). Luria-Bertani broth was used for all E. coli cultures, and when required, antibiotics were added to the culture at the following concentrations: kanamycin, 50 μg/ml; carbenicillin, 50 μg/ml; chloramphenicol, 34 μg/ml.

Chemicals and reagents. [14C]Sodium bicarbonate (50 Ci · mol⁻¹) was purchased from American Radiolabeled Chemicals, Inc. All other chemicals were...
purchased from Sigma and Fisher Scientific. All media were purchased from Difco. Nucleotide primers were synthesized by Integrated DNA Technologies, Inc.

**qPCR analysis.** Total RNA (5 μg) was treated with 2.5 units of DNase I (Ambion) according to the manufacturer’s instructions. First-strand cDNA was synthesized using SuperScript III and 6 μg of random primers (Invitrogen). The cDNA was purified with a QIAquick PCR purification kit (QIAGEN). The primers used are listed in Table 1. The primers corresponding to each ORF were designed to amplify less than 100 bp of product. Quantitative real-time PCR (qPCR) was performed in triplicate with three different concentrations of the diluted cDNAs (0.5 ng, 1 ng, and 5 ng) and 25 μl of Q SYBR Green Supermix (2× Premixture; Bio-Rad) in a total volume of 50 μl. The qPCR was carried out at 95°C for 20 s and 60°C for 20 s (40 cycles). Calculated thresholds were determined using the maximum-curvature approach after determination of per-well baseline cycles (iCycler; Bio-Rad). The amplified DNA samples were further subjected to melting-curve analysis and 1.2% agarose gel electrophoresis to verify the amplification product. Relative quantities of target ORF transcripts were calculated using the cycle threshold (Ct) values of the housekeeping gene, sigA (912 DANIEL ET AL. J. BACTERIOL.), according to the manufacturer’s recommendations. The aminooxy-dUTP-conjugated cDNA probes were spin dried (SpeedVac; Savant) and resuspended in 50 mM sodium carbonate buffer (pH 9.3) and cyanine monofunctional dyes (Amersham) for 1 h at room temperature, followed by quenching with 4 M hydroxylamine. The cyanine-labeled probes were hybridized again with a QIAquick PCR purification kit. All hybridizations were performed with dye reversal replicates. QuantArray (version 3.0; Perkin-Elmer) was used for 16-bit TIFF image quantification and initial data visualization. The hybridization signal was subjected to normalization and clustering by using an open-source R (version 2.1.1) package (http://www.bioconductor.org/).

**Recombinant-protein expression and preparation of cell extracts.** The ORF for β5 (accD6; Rv2247) was cloned by PCR using Phi Turbo HotStart DNA polymerase (Strategene) from the genomic DNA of *M. tuberculosis* H37Rv. The expression construct was prepared in pET 200 directional-TOPO expression vector (Invitrogen), and sequence integrity was confirmed by DNA sequencing. BL21 Star (DE3) host cells were transformed with the expression construct, and the overnight culture was diluted 1:50 in fresh medium. IPTG (isopropyl-β-D-thiogalactopyranoside) was added to a final concentration of 1 mM when the culture reached an optical density at 600 nm (OD600) of 0.7, and the induction was carried out for 4 h in a 37°C shaker. The α5 subunit was expressed and biotinylated with the *E. coli* biotin ligase as described previously (22). Following induction, the cells were washed and resuspended in lysis buffer (50 mM sodium phosphate, 300 mM NaCl, 1 mM phenylmethylsulfonyl fluoride). The cells were disrupted by sonication using a Branson Sonifier 450 (Branson Ultrasonics Corp.). The cell lysates were clarified by centrifugation at 12,000 × g, and the supernatants were used for purification of the expressed proteins. The β5 (Rv3280) and e (Rv3281) subunits were expressed in *E. coli* as described previously (22).

**Purification, SDS-PAGE analysis, and dialysis of expressed proteins.** The clear supernatants obtained from the above-mentioned lysates were applied to TALON cobalt affinity resin (BD Biosciences), and the His-tagged β5 and α5 (after biotinylation) were purified by the batch/gravity procedure of the manufacturer with the following modifications: the bound proteins were washed with 10 mM imidazole and eluted from the affinity resin with 100 mM imidazole and 500 imidazole elution steps. The eluted fractions were analyzed with an 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and fractions containing pure protein were pooled and dialyzed against 100 mM potassium phosphate (pH 8.0) containing 10% glycerol at 4°C. The β5 and e subunits were purified as described previously (22).

**Reconstitution of the carboxylase complex.** Following dialysis, the purified α5 subunit (100 μM/ml) was mixed with the purified β5 subunit (100 μM/ml), and the purified α5, β5, and e subunits were mixed together in a 1:1.2 molar ratio and incubated on ice for 6 h. Following this, the solutions were concentrated four- to

| Gene (ORF no.) | Primer sequence<sup>a</sup> | Product size (bp) | Reference |
|----------------|-----------------------------|-------------------|-----------|
| accA1 (Rv2501c) | F: 5′-GCGTGGCACTATGGTCAAAGAATT-3′<br>R: 5′-CCGAGCTTACACACCACCGGCTACA-3′ | 54 | This study |
| accA2 (Rv0973c) | F: 5′-GCGTGGCACTATGGTCAAAGAATT-3′<br>R: 5′-CCGAGCTTACACACCACCGGCTACA-3′ | 86 | This study |
| accA3 (Rv3285) | F: 5′-GCGTGGCACTATGGTCAAAGAATT-3′<br>R: 5′-CCGAGCTTACACACCACCGGCTACA-3′ | 57 | This study |
| accD1 (Rv2050c) | F: 5′-GCGTGGCACTATGGTCAAAGAATT-3′<br>R: 5′-CCGAGCTTACACACCACCGGCTACA-3′ | 67 | This study |
| accD2 (Rv0974c) | F: 5′-GCGTGGCACTATGGTCAAAGAATT-3′<br>R: 5′-CCGAGCTTACACACCACCGGCTACA-3′ | 85 | This study |
| accD3 (Rv0904c) | F: 5′-GCGTGGCACTATGGTCAAAGAATT-3′<br>R: 5′-CCGAGCTTACACACCACCGGCTACA-3′ | 92 | This study |
| accD4 (Rv3799c) | F: 5′-GCGTGGCACTATGGTCAAAGAATT-3′<br>R: 5′-CCGAGCTTACACACCACCGGCTACA-3′ | 67 | This study |
| accD5 (Rv3280) | F: 5′-GCGTGGCACTATGGTCAAAGAATT-3′<br>R: 5′-CCGAGCTTACACACCACCGGCTACA-3′ | 95 | This study |
| accD6 (Rv2247) | F: 5′-GCGTGGCACTATGGTCAAAGAATT-3′<br>R: 5′-CCGAGCTTACACACCACCGGCTACA-3′ | 76 | This study |
| accE (Rv3281) | F: 5′-GCGTGGCACTATGGTCAAAGAATT-3′<br>R: 5′-CCGAGCTTACACACCACCGGCTACA-3′ | 51 | This study |
| sigA (Rv2703) | F: 5′-GCGTGGCACTATGGTCAAAGAATT-3′<br>R: 5′-CCGAGCTTACACACCACCGGCTACA-3′ | 12 | This study |

<sup>a</sup> F, forward primer; R, reverse primer.
sixfold by ultracentrifugation in Centrifrep YM-10 filters (Millipore) at 700 × g for 4 h at 4°C and used in the carboxylase assay.

Acyl-CoA carboxylase assay. The carboxylase activity of the reconstituted ασ-βε complex was measured by following the incorporation of the radiolabel from NaH14CO3 into acid-stable reaction product using a modified procedure of Hunaiti and Kolattukudy (15). The reaction was carried out in a 100-μl volume containing 100 mM potassium phosphate, pH 8.0, 300 μg bovine serum albumin, 3 mM ATP, 5 mM acetyl-CoA or propionyl-CoA, 5 mM NaH14CO3, 5 mM MgCl2, and 20 to 30 μg of reconstituted enzyme. Following incubation at 30°C for 2 h, the reaction was stopped with 150 μl of 6 M HCl, and the entire solution was evaporated to dryness in a heating block at 100°C. The residue was resuspended in 100 μl water, and the radioactivity was measured by liquid scintillation counting. The effects of inhibitors were determined by preincubating the reconstituted ασ-βε complex with the inhibitor for 10 min at 24°C, after which the percent inhibition above the respective solvent control was determined. The kinetic parameters for the ασ-βε carboxylase were calculated using nonlinear regression analysis with the Michaelis-Menten equation, and the 50% inhibitory concentrations (IC50s) were determined from the sigmoidal dose-response equation (GraphPad Prism version 3.02; GraphPad Software).

Microarray accession number. The data discussed in this publication have been deposited in NCBI’s Gene Expression Omnibus (GEO [http://www.ncbi.nlm.nih.gov/geo/]) and are accessible through GEO Series accession number GSE5977.

RESULTS

Analysis of carboxylase subunit transcript levels. To determine which of the multiple carboxylase subunit genes are actually expressed in M. tuberculosis, we analyzed the expression levels of all of the ORFs encoding the carboxylase subunits by qPCR and microarray analyses. RNA isolated from cells at exponential growth phase was subjected to qPCR, and each C_T value was normalized to the level of the internal control sigA gene expression in exponentially growing cultures of M. tuberculosis. Total RNA was prepared from mid-log-phase cultures of M. tuberculosis H37Rv (OD_600 = 0.5). The transcript level of each acyl-CoA carboxylase subunit is indicated relative to the expression level of sigA as an internal control. Values represented are means and standard errors.

FIG. 1. Quantitative real-time PCR analysis of acyl-CoA carboxylase gene expression in exponentially growing cultures of M. tuberculosis. Total RNA was prepared from mid-log-phase cultures of M. tuberculosis H37Rv (OD_600 = 0.5). The transcript level of each acyl-CoA carboxylase subunit is indicated relative to the expression level of sigA as an internal control. Values represented are means and standard errors.

The gene numbers and the amino acid identities of the respective AccD6 subunits are indicated. AccA3, acetyl/propionyl-CoA carboxylase (ασ subunit); AccD6, acetyl/propionyl-CoA carboxylase (βε subunit); FabD, malonyl-ACP transacylase.

FIG. 2. Expression levels of carboxylase subunits during normal M. tuberculosis growth. Wild-type M. tuberculosis was grown in 7H9 complete medium (pH 7.0; 37°C) under normal aeration in a rolling culture tube. The cells were harvested at OD_600 of 0.53 (after 6 days of inoculation; “Mid-log”), 1.17 (after 8 days; “Late-log”), and 1.42 (after 12 days; “Stationary”). The total RNA was isolated and hybridized on DNA oligonucleotide microarrays as described in Materials and Methods. The hybridization signals were subjected to scale print tip median absolute deviation and quantile normalization. The relative expression level change for each acyl-CoA carboxylase subunit was obtained by comparing mid-log growth phase as a reference signal at late-log and stationary growth phases. The error bars indicate standard deviations.

FIG. 3. Genetic locus of AccD6 (βε) in pathogenic mycobacteria. The gene numbers and the amino acid identities of the respective AccD6 subunits are indicated. AccA3, acetyl/propionyl-CoA carboxylase (ασ subunit); AccD6, acetyl/propionyl-CoA carboxylase (βε subunit); FabD, malonyl-ACP transacylase.

FIG. 4. SDS-PAGE analysis of FabD and mycobacterial mycolic acid synthase complex. FabD, MALONYL-ACP transacylase. AccD6, Acyl-CoA carboxylase.
of the acyl-CoA carboxylase. The ε and β₆ subunits were purified as described previously (22).

Reconstitution and characterization of the enzymatic activity of α₅β₆ carboxylase. Following dialysis, the α₅ and β₆ subunits were mixed together in a 1:1 molar ratio, and the reconstituted α₅β₆ complex was assayed for acyl-CoA carboxylase activity. The α₅β₆ complex carboxylated acetyl-CoA with high specific activity (1.1 nmol/mg/min) and propionyl-CoA at lower levels (0.36 nmol/mg/min). The activity increased linearly with time and protein concentration (Fig. 5A and B) and displayed typical Michaelis-Menten kinetics for bicarbonate (Fig. 5C), acetyl-CoA and propionyl-CoA (Fig. 5D), and ATP (Fig. 5E). Under the assay conditions, saturation was reached with 2 mM bicarbonate, 5 mM acetyl-CoA or 2.5 mM propionyl-CoA, and 0.5 mM ATP. The optimal MgCl₂ concentration with 2 mM bicarbonate, 5 mM acetyl-CoA or 2.5 mM propionyl-CoA, and 0.5 mM ATP. The optimal MgCl₂ concentration was 10 mM for acetyl-CoA carboxylase (ACC) activity and 5 mM for propionyl-CoA carboxylase (PCC) activity. The kinetic parameters for the α₅β₆ complex are tabulated in Table 2.

Effects of inhibitors on α₅β₆ carboxylase. The carboxylase activities of α₅β₆ were inhibited by avidin and several other inhibitors. As shown in Table 3, at 100 μM, dimethyl itaconate and C75 inhibited the ACC activity of α₅β₆ by 96% and 87%, respectively, and the PCC activity by 53% and 54%, respectively. Haloxypin, cerulenin, and 1,2-cyclohexanediene inhibited the enzymatic activities significantly. The dose dependence of inhibition by dimethyl itaconate and C75 of the ACC and PCC activities of α₅β₆ was investigated, and the results are shown in Fig. 6. From this sigmoidal dose-response analysis, the IC₅₀ values for dimethyl itaconate were calculated to be 8.2 μM for ACC and 10.8 μM for PCC. For C75, the IC₅₀ were 50.2 μM for ACC and 101.5 μM for PCC.

Effect of the epsilon subunit on the α₅β₆ complex and stoichiometry of the α₅β₆ carboxylase. We examined the effect of the epsilon (ε) subunit on the activity of the α₅β₆ complex. In our previous report on the effect of the ε subunit on the carboxylase activities of the α₅β₆ complex, we determined that the optimal molar ratio of α₅ to β₆ to ε was 1:1:2 (22). Therefore, we incubated the α₅, β₆, and ε subunits together at the same molar ratio of 1:1:2 prior to assay. As shown in Fig. 7, the ε subunit inhibited both the ACC and PCC activities of the α₅β₆ complex. However, the ε subunit stimulated the ACC and PCC activities of the α₅β₆ complex, as reported previously (reference 22 and data not shown). We analyzed the stoichiometry of the reconstituted α₅β₆ carboxylase by gel filtration chromatography on Sepharose CL-6B (a 1.100-mm by 5-mm column equilibrated with 50 mM potassium phosphate buffer, pH 7.0, and 50 mM NaCl). The reconstituted α₅β₆ complex eluted in a peak with the apparent molecular mass of a dodecamer comprising six molecules each of the α₅ and β₆ subunits (data not shown).

DISCUSSION

Mycolic acid biosynthesis in M. tuberculosis involves FAS I, which carries out the de novo synthesis of C₁₄-C₂₆ fatty acids (16), and the FAS II multisubunit complex, which is incapable of de novo fatty-acid biosynthesis but elongates C₁₄ and C₁₆ primes to produce meromycolates (28). The FAS II complex in M. tuberculosis is located in a genetic locus that also contains a one, uncharacterized acyl-CoA carboxylase carboxyltransferase subunit, accD6 (β₆) (6), which could provide malonyl-CoA to the β-ketoacyl-ACP synthase and to FAS I. Malonyl-CoA is converted to malonyl-ACP by the malonyl-CoA-ACP transacylase (FabD) (19) and is utilized by the β-ketoacyl-ACP synthases (KasA/KasB) (5, 18, 27) in successive reactions with the other enzymes of the FAS II complex to produce the meromycic acids. Protein-protein interaction analyses have shown that FabD is a structural component of the FAS II complex (30) and that KasA, KasB, InhA, MabA (FabG1), and FabH (β-ketoacyl-ACP synthase III) interact with each other (31).

The reconstituted α₅β₆ acyl-CoA carboxylase, reported here, preferred acetyl-CoA over propionyl-CoA, in contrast to the α₅β₆ complex, which preferred propionyl-CoA over acetyl-CoA (10, 22). This observation leads us to suggest that M. tuberculosis may utilize the β₆ subunit with α₅ to provide malonyl-CoA to FAS I and to the FAS II complex for de novo fatty-acid biosynthesis and mycolic acid biosynthesis, respectively, and the β₆ subunit with α₅ for providing methylmalonyl-CoA for branched-fatty-acid biosynthesis. The rate of acetyl-CoA carboxylation by the α₅β₆ complex increased when the ε subunit was bound to it (10, 22), and thus, the α₅β₆-ε complex could also provide malonyl-CoA for fatty-acid biosynthesis in mycobacteria. The α₅ subunit also interacts with the β₆ (AccD4) subunit, which is possibly involved in the carboxylation of fatty acids that may then be incorporated into mycolic acids (11, 22, 24).

Isoniazid was shown to inhibit the biosynthesis of mycolic acids (29), and the reductases involved in fatty-acid elongation isolated from Mycobacterium avium were shown to be selectively inhibited by the drug (17). More recent work has shown that the reductases are the targets of isoniazid inhibition in M. tuberculosis (1, 25). In isoniazid-treated M. tuberculosis cul-
The accD6 ORF was upregulated, along with the other FAS II complex members in its neighborhood, which have been shown to be involved in mycolic acid biosynthesis (33).

The β6 subunits of other pathogenic mycobacteria, *Mycobacterium leprae* and *Mycobacterium bovis*, show a high degree of identity (93% and 100%, respectively) with the *M. tuberculosis* α3-β6 complex.

### TABLE 2. Kinetic parameters for the acyl-CoA carboxylase α3-β6 of *M. tuberculosis*

| Substrate      | ACC          | PCC          |
|----------------|--------------|--------------|
|                | $V_{\text{max}}$ | $K_m$ | SC | $V_{\text{max}}$ | $K_m$ | SC |
| Acetyl-CoA     | 1.1          | 4.9        | 0.22 | NA | NA | NA |
| Propionyl-CoA  | NA           | NA         | 0.36 | 0.97 | 0.37 |
| NaHCO₃         | 0.61         | 0.68       | 0.9  | 0.43 | 0.66 | 0.65 |
| ATP            | 0.67         | 0.12       | 5.6  | 0.34 | 0.06 | 5.67 |

*V_{\text{max}}$, mmol/mg/min; $K_m$, mM; SC, specificity constant ($V_{\text{max}}/K_m$); NA, not applicable.

### TABLE 3. Effects of inhibitors on the carboxylase activities of the reconstituted *M. tuberculosis* α3-β6 complex

| Inhibitor                              | ACC | PCC |
|----------------------------------------|-----|-----|
| Avidin                                 | 93 ± 0.1 | 94 ± 0.7 |
| Dimethyl itaconate                     | 96 ± 5  | 53 ± 4 |
| C75                                    | 87 ± 16 | 54 ± 12 |
| Haloxyfip                              | 60 ± 4  | 53 ± 8 |
| Cerulein                               | 54 ± 20 | 31 ± 10 |
| 1,2-Cyclohexanedione                   | 40 ± 23 | 45 ± 11 |
| Diclofop                               | 13 ± 3  | 9 ± 6 |
| Alloxymid                              | 11 ± 5  | 20 ± 9 |
| 5-(Tetradecyloxy)-2-furoic acid        | 8 ± 0.7 | 0 |
| 5-Iodotubericidin                      | 0     | 28 ± 5 |

*The α3-β6 complex was preincubated with 1 unit avidin (binds 1 μg d-biotin) or 100 μM inhibitor and used in the assay. Percent inhibition above the respective solvent control is given as mean ± standard deviation from three independent assays of the carboxylase with inhibitor.*
Catalytic activities of M. leprae ORFs in its genome (7). Interestingly, the ε unit indicated that the ε subunit is essential for the mycobacterium during the late-log and stationary growth phases, suggesting that these carboxylase subunits may be less important for the mycobacterium during the late-log and stationary growth phases. The inhibition of the carboxylase activities of the α3β6 complex when reconstituted with the ε subunit indicated that the ε subunit hindered interaction between the α3 and β6 subunits. Analysis of the stoichiometry of the α3β6 complex indicated that it is reconstituted as a dodecamer containing six molecules each of the α3 and β6 subunits, similar to the α3β2 complex, which was reported to exist as a dodecamer (10).

Inhibitors that were previously shown to target a particular enzyme have subsequently been demonstrated to inhibit novel targets. C75 is a synthetic inhibitor of fatty acid synthase and is a potential antiobesity and antitumor drug (20). C75-CoA was recently shown to inhibit carnitine palmitoyl transferase I in a novel mode of action (3). In another example, itaconate, which is a potent inhibitor of isocitrate lyase, was shown to inhibit propionyl-CoA carboxylase in cell extracts of Rhodospirillum rubrum (4). Isocitrate lyase is a glyoxylate shunt pathway enzyme that is used by M. tuberculosis to utilize fatty acids as a carbon source (21). Therefore, we investigated the effects of several inhibitors that have been shown to inhibit fatty-acid metabolism but had not been shown to inhibit ACC activity. Interestingly, we found that C75 and dimethyl itaconate severely inhibited the carboxylase activities of α3β6, in our assays (Fig. 6). Further work on the mechanism of action of these inhibitors is needed, and the characterization of such inhibitors, which target multiple biochemical pathways, may lead to the identification of potent drug candidates against M. tuberculosis.

FIG. 6. Inhibition of acyl-CoA carboxylase activities of α3β6 by dimethyl itaconate and C75. Inhibitions by dimethyl itaconate of ACC (■) and PCC (□) activities (A) and by C75 of ACC (▲) and PCC (▲) activities (B) were measured after preincubation of the respective inhibitor at the indicated concentrations with reconstituted α3β6 for 10 min at 24°C prior to the carboxylase assay. Percent inhibition above the solvent (dimethyl sulfoxide) control was plotted as the mean with standard deviation.

FIG. 7. Effect of the epsilon subunit on the α3β6 carboxylase. The purified α3 and β6 subunits were preincubated together in a 1:1 molar ratio, and the α3, β6, and ε subunits were preincubated together in a 1:1:2 molar ratio on ice for 6 h prior to a carboxylation assay with acetyl-CoA or propionyl-CoA. Values represented are means and standard errors.

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