Kinetic and Structural Analysis of a New Group of Acyl-CoA Carboxylases Found in *Streptomyces coelicolor* A3(2)*

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Two acyl-CoA carboxylases from *Streptomyces coelicolor* have been successfully reconstituted from their purified components. Both complexes shared the same biotinylated α subunit, AccA2. The β and the ε subunits were specific from each of the complexes; thus, for the propionyl-CoA carboxylase complex the β and ε components are PceB and PceE, whereas for the acetyl-CoA carboxylase complex the components are AccB and AccE. The two complexes showed very low activity in the absence of the corresponding β subunits; addition of PceE or AccE dramatically increased the specific activity of the enzymes. The kinetic properties of the two acyl-CoA carboxylases showed a clear difference in their substrate specificity. The acetyl-CoA carboxylase was able to carboxylate acetyl-, propionyl-, or butyryl-CoA with approximately the same specificity. The propionyl-CoA carboxylase could not recognize acetyl-CoA as a substrate, whereas the specificity constant for propionyl-CoA was 2-fold higher than for butyryl-CoA. For both enzymes the ε subunits were found to specifically interact with their carboxyltransferase component forming a β-ε subcomplex; this appears to facilitate the further interaction of these subunits with the α component. The ε subunit has been found genetically linked to several carboxyltransferases of different *Streptomyces* species; we propose that this subunit reflects a distinctive characteristic of a new group of acyl-CoA carboxylases.

The first committed step in the biosynthesis of long-chain fatty acids in all animals, plants, and bacteria is catalyzed by acetyl-CoA carboxylase (ACC)† (EC 6.4.1.2) (1). The reaction catalyzed by ACC involves two separate reactions.

1. HCO$_3$ + Mg-ATP + enzyme-biotin \( \rightarrow \) enzyme-biotin-CO$_2$ + Mg-ADP + P$_i$

2. Enzyme-biotin-CO$_2$ + acetyl-CoA \( \rightarrow \) enzyme-biotin + malonyl-CoA

SCHEME I

ACC is composed of three different components, which allow it to carry out these two distinct reactions. The BC component catalyzes the first half-reaction which involves the phosphorylation of bicarbonate by ATP to form a carboxylphosphate intermediate, followed by transfer of the carboxyl group to biotin to form carboxybiotin. *In vivo*, biotin is attached to the 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. In the second reaction, catalyzed by carboxyltransferase, the carboxyl group is transferred from biotin to acetyl-CoA to form malonyl-CoA.

Animals contain all three of these components in one polypeptide chain. In contrast, three different proteins compose the *Escherichia coli* and *Bacillus subtilis* form of ACC (2, 3). *E. coli* ACC has long served as model system for mechanistic studies of biotin-dependent carboxylases (4–7). However, no other bacterial ACC have been studied in detail.

Interestingly, several complexes with ACC activity have been purified from a number of actinomycetes and other bacteria. These complexes also possess the ability to carboxylate other substrates, including propionyl- and butyryl-CoA (8–10). Consequently, these enzymes are referred to as acyl-CoA carboxylases, and all of them consist of two subunits, a larger one (the α-chain) with the ability to carboxylate its covalently bound biotin group, and a smaller subunit (the β-chain) bearing the carboxyltransferase activity. Little is known about the biochemistry and physiological role of these enzymes, and so far all of these complexes have shown, at least *in vitro*, higher affinity by propionyl-CoA compared with acetyl- or butyryl-CoA; therefore, many of them are called PCCs (EC 6.4.1.3). The quaternary structure of the acyl-CoA carboxylase complexes has been reported only in a few organisms, such as *Mycobacterium smegmatis* (α$_{90}$β$_{90}$) (11), *Sacharopolyspora erythraea* (α$_{90}$β$_{90}$) (9), and *Myxococcus xanthus* (α$_{90}$β$_{90}$) (12), and in all cases the stoichiometry of the complex subunits was 1:1.

Attempts to identify enzymes with acyl-CoA carboxylase activity in *S. coelicolor* led to the characterization of three complexes. The first one, purified by Bramwell *et al.* (13), showed specificity for propionyl-CoA; therefore, it was classified as a PCC. This enzyme consisted of a biotinylated protein, PccA, of 88 kDa, and a non-biotinylated component, the carboxyltransferase, of 66 kDa. In our laboratory, two new carboxylases from *S. coelicolor* have been partially characterized at both the genetic and biochemical levels (14, 15). One of them was defined...
as a PCC, because a mutation in its carboxyltransferase component, pccB, affected only PCC activity levels (14). This enzyme appeared to be formed by two subunits: the biotinylated α subunit, encoded by either of the two identical genes named accA1 and accA2, and the carboxyltransferase component, encoded by pccB. The third acyl-CoA carboxylase characterized was shown to be essential for S. coelicolor viability, and it was called ACC (15). An interesting characteristic of this complex was that a third subunit (ε), AccE, considerably enhanced the basal activity obtained by the α and β subunits, encoded by AccA2 and AccB, respectively.

Little is known about the physiological role of acyl-CoA carboxylases in bacteria; although the PCC activity of these complexes should certainly be involved in the catabolic pathway of odd-chain fatty acids and branched-chain amino acids (16). In Streptomyces we were able to demonstrate, by constructing a conditional mutant in its carboxyltransferase component, AccB, that the main physiological role of the ACC complex was that of an ACC that synthesizes malonyl-CoA for both fatty acid and polyketide biosynthesis (15). Because polyketide synthetases are large multienzyme systems that are responsible for the stepwise biosynthesis of complex natural products from simple 2-, 3-, and 4-carbon building blocks such as acetyl-, propionyl-, and butyryl-CoA and their activated derivatives malonyl-, methylmalonyl-, and ethylmalonyl-CoA (17, 18), it is obvious that the acyl-CoA carboxylases will certainly play a fundamental role for the biosynthesis of this pharmacologically important compounds (15).

In this paper we describe the kinetic properties of the two acyl-CoA carboxylases, ACC and PCC, previously described by our group (14, 15). We also present data that address the functional significance of the ε subunit in both enzyme complexes and propose a new group of enzyme within the acyl-CoA carboxylases.

### EXPERIMENTAL PROCEDURES

#### Bacterial Strains, Culture, and Transformation Conditions

E. coli strain DH5α was used for routine subcloning and was transformed according to Sambrook et al. (19). Transformants were selected on media supplemented with the appropriate antibiotics: 100 μg ml⁻¹ ampicillin, 25 μg ml⁻¹ chloramphenicol, or 30 μg ml⁻¹ kanamycin. Strain BL21 λ(DE3) is an E. coli B strain lysogenized with λDE3, a prophage that expresses the T7 RNA polymerase from the IPTG-inducible lacUV5 promoter (20). Rosetta λ (DE3) expresses rare tRNAs to facilitate expression of genes that encode rare E. coli codons. ET 12567 was used for E. coli-Streptomyces intergeneric conjugations. Strains genotypes and recombinant plasmids are listed in Table I.

#### Growth Conditions, Protein Production, and Preparation of Cell-free 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 medium in the presence of the corresponding antibiotics for plasmid maintenance. To improve the biotinylation of AccA2 in E. coli, the strains containing pTR204 were also transformed with pCY216 (23), which overexpresses the E. coli biotin ligase (BirA); 10 μM t-biotin was also added to the medium. Overnight cultures were diluted 1:100 in fresh medium and grown to an OD₆₀₀ of 0.8 before the addition of IPTG to a final concentration of 0.1 mM. Induction was allowed to proceed for 8 h. The cells were harvested, washed and resuspended in buffer A (50 mM Tris-HCl, pH 8, 300 mM NaCl, 0.75 mM DTT, 1 mM EDTA, 10% glycerol).

#### Protein Methods

Cell-free extracts and purified proteins were analyzed by SDS-PAGE (24) using a Bio-Rad mini-gel apparatus. The final acrylamide monomer concentration was 8, 10, or 18% (w/v) for the separating gel and 5% for the stacking gel. Analytical gel electrophoresis of the native enzyme under denaturing conditions was carried out at 4 °C in a 6% polyacrylamide gel with Tris-glycine buffer, pH 8.3, for 2 h. Alternatively, gels were soaked in 60 mM Tris-HCl at pH 9.4 and pre-run for 60 min before samples were loaded. Coomassie Brilliant Blue was used to stain protein bands. The biotinylated proteins were

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**TABLE I**

| Strains and plasmids used |
|---------------------------|
| **Strain/plasmids** | **Description** | **Reference/source** |
| E. coli DH5α | F⁻ ΔfplomaΔZAM15 ΔlacsZYA-argF) U169 endA1 recA1 hsdR17 deoR supE44 thi-1 gyrA96 relA1 | Novagen (21) |
| BL21 λ(DE3) | F⁻ ompT rBi⁻ mB⁻ (DE3) | Novagen (20) |
| Rosetta λ (DE3) | pLacF+Cm' expresses rare tRNAs, facilitates expression of genes that encode rare E. coli codons | Novagen |
| ET 12567 | superE44 hsdR17 (rB B-) ara-14 pro A2 lacY galK2 rpsL20 xyl-5 mtl-1 dam - lac - dcm - hsdM-Cm' | Novagen (22) |

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**Strains and plasmids used**

| Strain/plasmids | Description | Reference/source |
|-----------------|-------------|-----------------|
| **Plasmids** | | |
| pGEM-T Easy | Used for cloning PCR products | Promega |
| pET22b(+) | Phagemid vector (Ap' lacZ') for expression of recombinant proteins under control of strong T7 transcription and translation signals | Novagen |
| pET28a(+) | Phagemid vector (Km' lacZ') for expression of recombinant proteins under control of strong T7 transcription and translation signals | Novagen |
| pCY216 | Vector containing E. coli birA gene | Novagen (23) |
| pET22b(+) | with pccB under control of strong T7 transcription and translation signals | Novagen (14) |
| pTR71 | pLJ2926 with pccB under the control of the lac promoter | Novagen (14) |
| pTR90 | pET22b(+) with accB under control of strong T7 transcription and translation signals | Novagen (15) |
| pTR88 | pET22b(+) with accBE under control of strong T7 transcription and translation signals | Novagen (15) |
| pTR116 | pET28a(+) with an insert carrying accBE His tag fusion gene under control of strong T7 transcription and translation signals | This work |
| pTR131 | pET28a(+) with an insert carrying pccB His tag fusion gene under control of strong T7 transcription and translation signals | This work |
| pTRL1 | pET28a(+) with an insert Ndel-XhoI carrying accB His tag fusion gene under control of strong T7 transcription and translation signals | This work |
| pTRK1 | pET28a(+) with pccB under control of strong T7 transcription and translation signals | This work |
| pTRK3 | pET28a(+) with an insert Ndel-EcoRI carrying pccB His tag fusion gene under control of strong T7 transcription and translation signals | This work |
| pTR237 | pET28a(+) with an accE His tag fusion gene under control of strong T7 transcription and translation signals | This work |
detected by modification of Western blotting procedure described by Nikolau et al. (25). After electrophoretic separation, proteins were electroblotted onto nitrocellulose membranes (Bio-Rad) and probed with alkali phosphatase-streptavidin conjugate (AP-streptavidin diluted 1:10,000) (Bio-Rad). Immunoblotting was performed according to Burnette (26) using anti-PccB or anti-AccB, in a 1:10,000 dilution of biotin anti-anti-Ab. In all cases, visualization was accomplished with an alkali phosphatase-tagged secondary antibody. Antisera against PccB, PccE, AccB, and AccE were elicited in rabbits following conventional procedures (26). Protein contents were determined by the method of Bradford (27) or Lowry with BSA as standard.

**ACC/PCP Assay: Radioactive Method—ACC and PCC activities in cell-free extracts and in *in vitro* reconstituted complexes obtained by mixing purified proteins were measured following the incorporation of radioactive 
\( ^{14} \)CO\(_2 \) into acid non-volatile material (9, 13). The reaction mixture contained 100 mM potassium phosphate, pH 8.0, 300 \( \mu \)g of BSA, 3 mM ATP, 5 mM MgCl\(_2\), 50 mM NaH\(_2\)CO\(_3\) (specific activity 200 \( \mu \)Ci mmol\(^{-1}\) (740 kBu mmol\(^{-1}\)), 0.5 mm substrate (acyetyl-CoA or propionyl-CoA), and 100 \( \mu \)g of cell-free protein extract in a total reaction volume of 100 \( \mu \)l. The reaction was initiated by the addition of NaH\(_4\)CO\(_3\), allowed to proceed at 30°C for 15 min, and stopped with 200 \( \mu \)l of 6 N HCl. The contents of the tubes were then evaporated to dryness at 95°C. The residue was resuspended in 100 \( \mu \)l of water, 1 ml of Op- tiphase liquid scintillation medium (Wallac Oy) was added, and 14°C radioactivity determined in a Beckman scintillation liquid counter. Nonspecific CO\(_2\) fixation by crude extracts was assayed in the absence of substrate. One unit of enzyme activity catalyzed the incorporation of 1 \( \mu \)mol of \( ^{14} \)CO\(_2\) into acid-stable products/min.

**Coupled Enzyme Assay—The rate of ATP hydrolysis by biotin carboxylase was measured spectrophotometrically (29).** The production of ATP was coupled to pyruvate kinase and lactate dehydrogenase, and the oxidation of NADH was followed at 340 nm. Assay were performed either in a micropipet reader or a Varian spectrophotometer. In the first case, measurements were carried out in a volume of 0.2 ml in 96-well polycarbonate cuvettes. The assay mixture contained 5 units of pyruvate kinase, 10 units of lactate dehydrogenase, 0.5 mM phosphoenolpyruvate, 0.2 mM NADH, 5 mM MgCl\(_2\), 0.3 mg/ml BSA, 100 mM potassium phosphate, pH 7.6, 3 mM ATP, 50 mM NaHCO\(_3\), and the appropriate concentrations of acetyl-, propionyl-, or butyryl-CoA. Reaction rates were determined by addition of the different enzyme components. The amount of coupling enzymes was sufficient to ensure that the initial velocity varied linearly with enzyme concentration. Data were collected using a Dynex MRX microplate reader interfaced to a PC equipped with a data acquisition program. Temperature was maintained at 30°C by circulating water bath with the capacity to heat and cool the thermoset of the cell compartment. Measurements in a spectrophotometer were conducted at 340 nm at room temperature (0.7 mOD units) except ATP and KHCO\(_3\). Cuvettes were preincubated at 37°C for 10 min. Oxidation of NADH was then recorded after addition of ATP until a stable base line was obtained, and the reaction was started by addition of NaHCO\(_3\). Initial velocities are 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 \( \mu \)mol of the respective carboxylated CoA derivative or ADP/min under the assay conditions described. Essentially identical activities are measured by the \( ^{14} \)CO\(_2\) fixation method and by spectrophotometric method. Specific activity is expressed as units/mg of AccA.

**High Pressure Liquid Chromatography (HPLC)—ACC and PCC activities in cell-free extracts and in *in vitro* reconstituted complexes obtained by mixing purified proteins were measured following the incorporation of radioactive HCO\(_3\)\(^{-}\) into acid non-volatile material (9, 13). The reaction mixture contained 100 mM potassium phosphate, pH 8.0, 300 \( \mu \)g of BSA, 3 mM ATP, 5 mM MgCl\(_2\), 50 mM NaH\(_2\)CO\(_3\) (specific activity 200 \( \mu \)Ci mmol\(^{-1}\) (740 kBu mmol\(^{-1}\)), 0.5 mm substrate (acyetyl-CoA or propionyl-CoA), and 100 \( \mu \)g of cell-free protein extract in a total reaction volume of 100 \( \mu \)l. The reaction was initiated by the addition of NaH\(_4\)CO\(_3\), allowed to proceed at 30°C for 15 min, and stopped with 200 \( \mu \)l of 6 N HCl. The contents of the tubes were then evaporated to dryness at 95°C. The residue was resuspended in 100 \( \mu \)l of water, 1 ml of Op- tiphase liquid scintillation medium (Wallac Oy) was added, and 14°C radioactivity determined in a Beckman scintillation liquid counter. Nonspecific CO\(_2\) fixation by crude extracts was assayed in the absence of substrate. One unit of enzyme activity catalyzed the incorporation of 1 \( \mu \)mol of \( ^{14} \)CO\(_2\) into acid-stable products/min.

**DNA Manipulations**

Isolation of chromosomal and plasmid DNA, restriction enzyme digestion, and agarose gel electrophoresis were carried out by conventional methods (19, 30).
and induction and protein purification carried out as described above. The outcome of this column was evaluated by SDS-PAGE and Western blot.

The interaction of PccB (or AccB) with AccA2 was studied in vitro by mixing purified His6-PccB (or His6-AccB) and AccA2, with or without addition of purified His6-PccE (or His6-AccE). After the incubation for 2 h at 4 °C, the mixtures were passed through an avidin-Sepharose affinity column equilibrated and washed (10 volumes) with buffer A, and eluted with the same buffer containing 5 mM biotin. The outcome of this column was evaluated by SDS-PAGE and Western blot.

RESULTS

Determination of the Optimal Molar Ratio of the Subunits of ACC and PCC Complexes—In a recent publication we showed that a small protein subunit with a calculated molecular mass of 7 kDa, called AccE, was essential to obtain maximal activity of the ACC complex (15). The gene encoding for this small subunit is part of a two-gene operon and is located downstream of accB. Considering that PccB is the carboxyltransferase component of the PCC complex and that both ACC and PCC share the same biotinylated component, we searched for the existence of an ORF encoding for an AccE homologue downstream of pccB. Using the Frame program (31) we detected an ORF encoding for a putative protein with 33% of amino acid sequence identity with AccE; we called it PccE. This ORF was annotated later by the S. coelicolor genome sequencing project (www.sanger.ac.uk/Projects/S_coelicolor/) as a putative ORF. To reproduce, with the purified components, the stimulatory effect seen with AccE on the ACC activity (15), and to check whether PccE had a similar effect on the PCC complex, we carried out the enzyme assay of both enzyme complexes in the presence or absence of the ε subunits. Very low activity was detected when mixtures containing 0.32 μM [AccA2-PccB] or [AccA2-AccB] were assayed with saturating concentration (0.5 mM) of propionyl-CoA. When AccE or PccE were added in 10 molar excess respect to the α/β subunits, a clear stimulatory effect of the enzyme activity was observed for both complexes (data not shown). These results showed that in vitro the reconstitution of the ACC and PCC complexes with purified components was efficient and also confirmed that PccE, like AccE, was essential to reach maximal enzyme activity.

The core component of all the acyl-CoA carboxylases isolated so far is formed by the α and β subunits, and where studied the stoichiometry of the components was found to be 1:1 (9-12). Thus, the titration of the effects of PccE and AccE on their respective acyl-CoA carboxylases was performed in reaction mixtures containing equimolar amounts (0.32 μM) of [AccA2-PccB] or [AccA2-AccB] and saturating concentrations of the substrate (0.5 mM propionyl-CoA). The rate of product formation depended on PccE or AccE concentration. Within the limits of the assay, very low activity could be observed in the absence of PccE or AccE (Fig. 1, A and B). For PccE, data presented a best fit to a hyperbolic curve from which a Vmax of 1123 milliunits/mg AccA2 was obtained. Half-maximal velocity was reached at 0.52 μM PccE (molar ratio 1:6:1:1 for PccE/AccA2: PccB). For AccE, Vmax was 576 milliunits/mg AccE and the half-maximal velocity was reached at 0.20 μM (molar ratio 0.62:1:1 for PccE/AccA2: PccB). Saturation (more than 90% of maximal velocity) was achieved at molar ratios higher than 9:1 or 3:1 for PccE or AccE, respectively, with respect to either the α or the β subunit.

To define whether the ε subunits of the ACC and PCC complexes were interchangeable, we determined the levels of enzyme activity of each couple of α and β subunits in the presence of AccE or PccE. For the AccA2-PccB couple, only PccE was capable of stimulating the enzyme activity, whereas, for the AccA2-AccB couple, only AccE was shown to be active (Fig. 1, A and B, respectively). This result clearly showed the high specificity of the ε subunits for each of the complexes.

Kinetic Parameters of the ACC and PCC Complexes—The kinetic characterization of the complexes 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 acyl-CoA carboxylases were kinetically characterized in terms of Km, Vmax, and substrate specificity. Table II shows the values obtained for each parameter with three different acyl-CoA derivatives: acetyl-CoA, propionyl-CoA, and butyryl-CoA. The affinity of the ACC complex for the substrates tested was approximately the same independently of the acyl moiety of the derivative. However, the efficiency was observed for propionyl-CoA, which not only renders the lower Km but also the highest Vmax. The PCC complex was significantly more active with propionyl-CoA, rather than with butyryl-CoA, but no activity was observed with acetyl-CoA. These results were in coincidence with those reported by Rodriguez and Gramajo (14), when enzyme assays were carried out in crude extracts. In all cases the kinetics were hyperbolic.

Effect of the ε Subunits on the Physical Behavior of the Other Components of the Complexes—Considering that AccA2 is a common subunit for both the ACC and PCC complexes and having in mind the genetic linkage existent between the β and ε encoding genes, we worked on the hypothesis of a putative

Fig. 1. Effects of the ε subunits on the acyl-CoA carboxylases. Increasing concentrations of the PccE (●) and AccE (○) subunits were added to the assay media containing equimolar amounts (0.32 μM) of the α and β subunits of the PCC (A) and ACC (B) complexes. Results presented are the average of at least four independent experiments with a standard error that was within ±5% of the mean.
protein-protein interaction between the specific β and ε subunits. To test this hypothesis and also to learn about any other putative protein-protein interaction, protein mixtures containing different combinations of the ACC and PCC α, β, and ε subunits were analyzed on native PAGE (Fig. 2A). AccA2 migrated as a single band, although part of the protein formed high molecular mass aggregates visualized as a smear near the top of the gel. No interactions were observed either between AccA2 and PccE or AccA2 and PccB, at least based on the formation of new bands of different mobility in the native PAGE. However, as can be observed in this figure, a new protein band appeared when PccE and PccB were preincubated. Interestingly, from the three bands observed for PccB alone, which probably represent different oligomeric forms of the protein, only the upper one was shifted by the presence of PccE. Considering that PccE runs out of a 6% native gel (although it can be perfectly seen on a 15% gel), the formation of a new band suggests the interaction between PccB and PccE. To further explore this possibility, we isolated the putative PccB-PccE complex band from the native gel and run it into an SDS-PAGE. Fig. 2B shows that under denaturing conditions two proteins, with molecular mass corresponding to those of PccB and PccE, were readily detected. When the three subunits of the PCC complex were preincubated, the only new band observed corresponded to that seen for the PccB-PccE mix, suggesting that, at least under these conditions, the only protein-protein interaction that can be detected is that between PccB and PccE. Western blot analysis demonstrated that no α subunit was present in this complex (data not shown).

The interaction between the components of the ACC complex was assayed under the same conditions as for PCC. In this case, we were unable to detect the formation of a new complex in any of the protein combinations tested (data not shown). Attempts to reveal protein-protein interactions by changing the pH of the native gels were also unsuccessful.

Analysis of the PccB-PccE Interaction by Native Gel Electrophoresis—In an effort to study the kinetics of the PccB-PccE interaction, increasing amounts of His$_6$-PccE were added to a fixed amount (4 μg) of His$_6$-PccB and analyzed into a 6% native PA gel. Fig. 3A shows that increasing amounts of PccE resulted in a corresponding increase in the PccB-PccE complex. The amount of protein complex formed was determined by densitometry of a Coomassie-stained gel. Interestingly, a sigmoidal curve best fitted to the data measured, and from that we were able to determine that saturation (more than 90% of maximal amount of complex formed) occurs beyond a molar ratio of 18:1 (PccE:PccB) (Fig. 3B). Although the first points of the curve could have some error because of the low sensibility of the Coomassie staining, we obtained the same results by developing the protein complex by Western blot with anti-PccB antibodies (data not shown). The fact that a saturation curve can be visualized confirms that the β-ε interaction is highly specific and is not the result of the formation of amorphous aggregates.

To determine the stoichiometry of the PccB-PccE interaction, a protein band corresponding to the complex was cut from the native gel and run into an SDS-PAGE. Protein concentration was estimated by constructing standard curves obtained by running in the same gel known concentrations of PccB and PccE (data not shown). As already shown in Fig. 2B, the complex was formed by both proteins and the quantitation of each band by densitometry allowed us to determine that the ratio of PccE:PccB was 10:1.

Coexpression of the ε Subunits with Their Corresponding Carboxyltransferases Leads to Protein-Protein Interactions in Crude Extracts—An additional strategy to study the physical interaction between AccE and PccE with their corresponding carboxyltransferases AccB and PccB involved copurification experiments. For this, each specific pair of subunits was coexpressed in E. coli and one of the interacting subunits (either β or ε) was tagged on the NH$_2$ terminus with His$_6$. In this way, if an stable protein complex was formed, both subunits should copurify after using a Ni$^{2+}$-NTA column.

The physical interaction of AccB with AccE was studied using plasmid pTR116. This plasmid contains the accBE operon cloned in pET28b, in such a way that AccB, encoded by the first gene of the operon, is expressed as a His$_6$ fusion protein. Cultures of E. coli containing pTR116 were IPTG induced and crude extracts passed through a Ni$^{2+}$-NTA-agarose affinity column. After extensive washes the protein specifically bound was eluted with buffer containing 150 mM imidazole. Protein samples were run in an SDS-PAGE and, as is shown in Fig. 4A, a small polypeptide of ~7 kDa coeluted with His$_6$-AccB, confirming for the first time an interaction between these two subunits. The smaller protein band was confirmed to be AccE by immunoblot experiments (Fig. 4B).
The physical interaction of PccB with His6-PccE was evaluated by the coexpression of these gene products from plasmids pTRK1 and pTRK3, respectively, in E. coli Rosetta (DE3) pLac promoter. Crude extracts of the IPTG-induced cultures were passed through a nickel-NTA-agarose affinity column, and the outcome of this column was evaluated by SDS-PAGE. A protein band with the same molecular mass of PccB coeluted with His6-PccE (Fig. 5A). We confirmed the nature of PccB by Western blotting, using a polyclonal anti-PccB antibody (Fig. 5B). Nonspecific interaction of PccB by the Ni2+-NTA column was not detected in control experiments (data not shown).

These studies clearly show that the ε subunits of both complexes are able to interact with their corresponding carboxyltransferases. Moreover, the detection of these complexes in crude cell extracts is a hint that the protein-protein interaction might have occurred in vivo before cells were disrupted.

The ε Subunit Is Necessary for an Efficient α-β Interaction—The BC-BCCP subunit of the ACC and PCC complex catalyzes the first half-reaction of these enzyme complexes, in which the biotin is carboxylated to form carboxybiotin. To complete the reaction, the carboxyltransferase must contact the biotinylated subunit to transfer the carboxyl group from the biotin to the appropriate short-chain carboxylic acid. The interaction of these subunits appears to be very weak, at least in the complexes where an ε subunit appears to be involved. For instance, attempts to purify the ACC complex from the native source were unsuccessful2 and the only PCC isolated as an active complex from S. coelicolor comprises only two subunits (13), different from the α and β subunits of the PCC characterized by our group (14). To determine if an interaction between the α and β subunits, although weak, could be detected in vitro, we carried out copurification experiments using purified proteins and affinity columns.

The interaction between AccA2 and PccB was studied by mixing the purified proteins His6-PccB and AccA2, in the presence or absence of His6-PccE. The protein mix was then passed through an avidin-Sepharose affinity column and the elution fractions analyzed by Western blotting using antibodies against PccB. Fig. 6A shows that, when PccE was present in the protein mix together with PccB and AccA2, PccB coeluted with AccA2, although at levels only detectable by immunoblotting. When PccE was left out of the protein mix, no PccB copurified with AccA2 (Fig. 6B). When the interaction between AccA2 and AccB was studied in the presence or absence of His6-AccE, we obtained the same results (data not shown). These experiments clearly show that, for both the ACC and the PCC complexes, the presence of the specific ε subunit is essential to allow the interaction of the α and the β subunits.

DISCUSSION

The genetic characterization of two acyl-CoA carboxylases of S. coelicolor, ACC and PCC (14, 15), has provided important insights toward the pathways that lead to the generation of short-chain acyl-CoAs, the building blocks of polyketide compounds. In this study, the successful in vitro reconstitution of the ACC and PCC complexes from their purified components allowed us to carry out a primary characterization of these two enzymes complexes at a biochemical and structural level.

All the acyl-CoA carboxylases (usually defined as PCC), isolated from diverse sources such as mammalian (32–34), nematicides (35), and bacteria (9–13), comprise two types of subunits: the α subunit containing the BC domain and the carboxyltransferase β subunit. The two acyl-CoA carboxylases described in this study showed a distinctive characteristic with respect to those mentioned above, and this was the existence of a third subunit, called ε, the presence of which dramatically stimulates the specific activity of the enzyme complexes (Fig. 1, A and B). Thus, we propose that these two acyl-CoA carboxylases can be considered as a new group, which includes a third subunit ε in addition to the α and β, commonly found among this family of enzymes. To know whether this new group of acyl-CoA carboxylases was more broadly distributed within Streptomyces a search for AccE homologues was carried out in the GenBank database. The search highlighted two new ε subunits besides PccE; interestingly, both of them are located downstream putative carboxyltransferases. One (CAC37886) is 33% identical to AccE, belongs to S. coelicolor and is located in cosmid 1G7, close to a putative type I polyketide synthetase; the second homologue is Sim12 (AAL15590), which presents a 55% identity with AccE and is part of the simocyclinone cluster of S. antibioticus (36). Based on this result, the possibility that new ε subunits exist downstream of putative carboxyltransferases associated with antibiotic clusters was carefully searched. First of all we looked for homologues to the carboxyltransferase AccB in the data base, and this search revealed various putative carboxyltransferases associated with polyketide clusters. These were putative decarboxylase called PgaL (AAK57534) from a silent angucycline cluster of Streptomyces sp. PGA64, a decarboxylase LanP (AAD13544) from the

\footnote{2} L. Diaconovitch, S. Peiró, E. Rodriguez, and H. Gramajo, unpublished data.
landomycin cluster of *S. cyanogenus*, and a putative decarboxylase JadN (AAK01934) from the jadomycin cluster of *S. venezuelae*. By analyzing the nucleotide sequences located immediately downstream of the carboxyltransferases with BlastX, we found in each of them a putative ORF with homology to the AccE subunit. Although we will need to prove the functionality of the putative subunits on each complex, these results strongly suggest that these new group of acyl-CoA carboxylases are broadly distributed within *Streptomyces*.

The kinetic characterization of the ACC and PCC complexes showed unique characteristics in relation to their substrate specificity. The ACC complex showed relaxed substrate selectivity and the specificity constant ($V_{\text{max}}/K_m$) for acetyl-, propionyl-, and butyryl-CoA was almost the same (Table II). This is in contrast with all the other acyl-CoA carboxylases characterized so far, whose preferred substrate is propionyl-CoA. The primary role of the *S. coelicolor* ACC appears to be the generation of malonyl-CoA for the biosynthesis of fatty acids during the exponential phase and for the biosynthesis of actinorhodin during the stationary phase of growth (15). Thus, in *vivo*, the preferred substrate for this enzyme complex appears to be acetyl-CoA. Remarkably, the PCC complex showed no detectable activity with acetyl-CoA as a substrate. However, it efficiently carboxylated butyryl- and propionyl-CoA, although with a clear preference for the latter (Table II). Therefore, both enzyme complexes have the potentiality to carboxylate propionyl-CoA *in vivo*.

Recently, the heterologous expression of the *S. coelicolor* PCC complex in *E. coli* has allowed the biosynthesis of methylmalonyl-CoA, a metabolite not normally accumulated in this microorganism (36), and the production of the 6-deoxyerythronolide B when DEBS1, -2, and -3 were coexpressed in the same strain (37). The successful production of a polyketide compound in a heterologous host will depend not only on the
values obtained when testing the effects of PccE on the PCC (PccE:PccB). These values show a good correlation with the proteins form a complex with a probably stoichiometry of 10:1 PccB-PccE interaction by SDS-PAGE indicated that both pro-
states, will require further investigation. The analysis of the PccB or if it can bind, albeit with less affinity, to the lower mass PccB. Whether PccE only interacts with one oligomeric form of interacts with the larger of the three oligomeric forms shown by Fig. 6. Evidence of a AccA2-PccB interaction by copurification from an avidin-Sepharose column. Panel A, a mixture of AccA2 (20 µg), PccB (20 µg), and PccE (10 µg) was loaded onto an avidin-Sepharose column, washed with 100 mM phosphate buffer, pH 7.6, and eluted with the same buffer containing 5 mM ∆-biotin (elution fractions 1–5). Aliquots of the eluted fractions were run on an 10% SDS-PAGE, transferred to nitrocellulose, and probed with anti-PccB antibodies. Panel B, same as panel A, but PccE was omitted from the mixture.

efficient expression of the polyketide synthetases but also on the effective biosynthesis of the precursors (such as methylmalonyl-, propionyl-, and ethylmalonyl-CoA). Therefore, the knowledge of the kinetic parameters of the different acyl-CoA carboxylases is of central importance in the selection of the most appropriate complex to be used for such purposes. The effects of ε on the activity of the ACC and PCC complexes follow saturation kinetics, suggesting the existence of protein-protein interactions. Complete saturation occurs at a molar ratio of ~3:1:1 (AccE:AccA2:PccB) for the ACC complex and 10:1:1 (PccE:PccB) for the PCC complex. The studies described here clearly showed that the σ subunits, PccE and AccE, interact specifically with their corresponding carboxyltransferases PccB and AccB (Figs. 2–5). The PccB-PccE interaction observed in native gels suggests that PccE preferentially interacts with the larger of the three oligomeric forms shown by PccB. Whether PccE only interacts with one oligomeric form of PccB or if it can bind, albeit with less affinity, to the lower mass states, will require further investigation. The analysis of the PccB-PccE interaction by SDS-PAGE indicated that both proteins form a complex with a probably stoichiometry of 10:1 (PccE:PccB). These values show a good correlation with the values obtained when testing the effects of PccE on the PCC complex activity (Fig. 1A), where maximal activity of the complex was reached at a PccE:PccB molar ratio above 9:1. Interestingly the kinetics of the ε-β interaction followed a sigmoidal curve, and the saturation was reached at very high ratio of PccE:PccB (~18:1) (Fig. 3, A and B). These results are curious, considering that the effect of the ε subunit on the PCC complex activity followed a typical hyperbolic curve and that the saturation was reached at a 10:1 molar ratio of PccE:PccB. We could probably think that these differences are provoked by the presence of the α subunit and/or the substrates, which could change the affinity of the β-ε interaction. Attempts to investigate the quaternary structure of the PccB-PccE complex were carried out by using gel-filtration chromatography (data not shown). Under the running conditions used, we were unable to detect any recognizable peak that could correspond to a PccB-PccE complex, probably reflecting the lability of this protein-protein interaction when high dilutions of the proteins are used. Although the interaction between AccE and AccB could be observed in copurification experiments (Fig. 4, A and B), we were unable to quantify for the AccE-AccB interaction because no such complex was observed on native PAGE.

Although the α and β components of all the purified acyl-CoA carboxylases form a tight complex, the interaction of the α and β subunits of the S. coelicolor ACC and PCC complexes appears to be very weak. Our results have shown that for both complexes, ACC and PCC, the specific ε subunit is essential to detect any interaction between the α and β subunits (Fig. 6, A and B); we speculate that this interaction only occurs after the β-ε subcomplex has been formed. From all these experiments, it is evident that the ε subunit has a key role in maintaining an active complex structure. Whether it also intervenes in the regulation of this new group of acyl-CoA carboxylases remains to be determined.

To further understand the nature of the β-ε interaction, we analyzed the amino acid sequence alignments of the four ε subunits published. As shown in Fig. 7A, the most conserved region between these proteins occurs in their amino-terminal half, whereas toward the carboxyl end of the proteins there are almost no conserved amino acid residues. When the amino acid sequence alignment was carried out for the AccE and PccE pair (the only ε subunits functionally characterized so far), the difference in the amount of conserved amino acid residues between the amino and the carboxyl end of the proteins was
very clear (40%) of identity for the amino-terminal half and 22% of identity for the carboxyl end; see Fig. 7B). Because we proved that AccE and PccE are not functionally interchangeable, we could hypothesize that the carboxyl end of the ε subunits dictates the specificity of the β-ε interaction. Construction of hybrid ε subunits might help to understand this point in the future.

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