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Complex Formation and Regulation of *Escherichia coli* Acetyl-CoA Carboxylase

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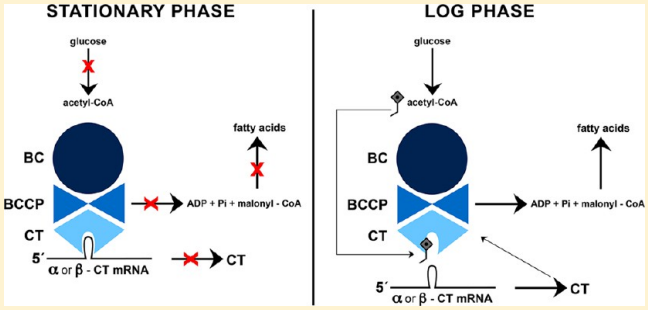
ABSTRACT: Acetyl-CoA carboxylase is a biotin-dependent enzyme that catalyzes the regulated step in fatty acid synthesis. The bacterial form has three separate components: biotin carboxylase, biotin carboxyl carrier protein (BCCP), and carboxyltransferase. Catalysis by acetyl-CoA carboxylase proceeds via two half-reactions. In the first half-reaction, biotin carboxylase catalyzes the ATP-dependent carboxylation of biotin, which is covalently attached to BCCP, to form carboxybiotin. In the second half-reaction, carboxyltransferase transfers the carboxyl group from carboxybiotin to acetyl-CoA to form malonyl-CoA. All biotin-dependent carboxylases are proposed to have a two-site ping-pong mechanism in which the carboxylase and transferase activities are separate and do not interact. This posits two hypotheses: either biotin carboxylase and BCCP undergo the first half-reaction, BCCP dissociates, and then BCCP binds to carboxyltransferase, or all three constituents form an enzyme complex. To determine which hypothesis is correct, a steady-state enzyme kinetic analysis of *Escherichia coli* acetyl-CoA carboxylase was conducted. The results indicated the two active sites of acetyl-CoA carboxylase interact. Both *in vitro* and *in vivo* pull-down assays demonstrated that the three components of *E. coli* acetyl-CoA carboxylase form a multimeric complex and that complex formation is unaffected by acetyl-CoA, AMPPNP, and mRNA encoding carboxyltransferase. The implications of these findings for the regulation of acetyl-CoA carboxylase and fatty acid biosynthesis are discussed.



Fatty acid biosynthesis is one of the most important and fundamental metabolic pathways in nature. Animals use fatty acids as a component of cellular membranes and for energy storage, while bacteria use fatty acids only for membrane biogenesis.[1] Thus, regulation of fatty acid biosynthesis is critical to the survival of organisms from bacteria to humans. Fatty acid synthesis in bacteria is commonly termed fatty acid synthesis II (FAS II) to distinguish it from the eukaryotic pathway, which is designated fatty acid synthesis I (FAS I). FAS II is distinguished from FAS I by the fact that all of the enzymes in the pathway are separate proteins; in FAS I, the enzymes constitute domains on a single polypeptide.[2] The major regulatory enzyme in bacterial fatty acid biosynthesis is acetyl-CoA carboxylase (ACC), which catalyzes the committed step in this pathway.[3]

Acetyl-CoA carboxylase catalyzes the biotin-dependent carboxylation of acetyl-CoA to form malonyl-CoA via two half-reactions (Scheme 1). In *Escherichia coli* and many other Gram-negative and Gram-positive bacteria, the enzyme consists of three different components: biotin carboxylase (BC), biotin carboxyl carrier protein (BCCP), and carboxyltransferase (CT). In the first half-reaction, biotin carboxylase catalyzes the formation of a reactive carboxyphosphate intermediate through the ATP-dependent phosphorylation of bicarbonate.[4] The carboxyl group is transferred from carboxyphosphate to the vitamin biotin, which *in vivo* is covalently attached to BCCP.[5,6] In the second half-reaction, carboxyltransferase transfers the carboxyl group from carboxybiotin to acetyl-CoA to make malonyl-CoA.[7,8]

While *E. coli* acetyl-CoA carboxylase consists of three different proteins, four different gene products are required to make the functional enzyme. The biotin carboxylase component is a homodimer with a monomer molecular mass of 49.4 kDa and is encoded by the *accC* gene. The BCCP component is a 16.7 kDa polypeptide that has been reported to form higher-molecular mass aggregates and is encoded by the *accB* gene.[9,10] The carboxyltransferase component is an $\alpha_2\beta_2$ heterotetramer with a 35.1 kDa α-subunit encoded by *accA* and a 33.2 kDa β-subunit encoded by *accD*.[11]

Acetyl-CoA carboxylase (like all biotin-dependent carboxylases) is considered to have a nonclassical or two-site ping-pong kinetic mechanism, which means the active sites for biotin carboxylase and carboxyltransferase are separate and do not interact or communicate with one another.[12] This type of kinetic mechanism is consistent with the fact that when either the carboxylase or transferase components of *E. coli* acetyl-CoA carboxylase are isolated, they retain their enzymatic activity only when free biotin (i.e., not attached to BCCP) is used as a substrate.[13] This observation has led to two hypotheses for the mechanism of action for the enzyme. One theory posits that

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Scheme 1. Acetyl-CoA Carboxylase Half-Reactions and Sum

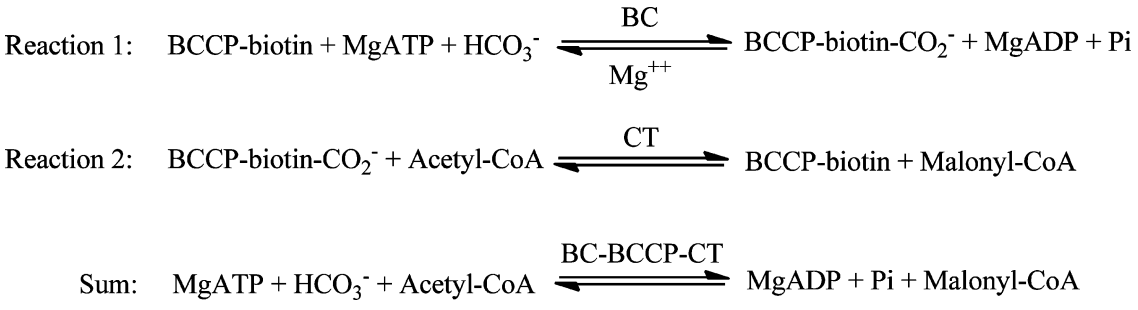

Table 1. Primers for Expression Plasmid Construction

primer	DNA sequence[a]	plasmid	enzyme
FlagBC5	5′-CTTCTTCATATG**GATTATAAAGACGATGACGATAAAA**TGCTGGATAAAATTGTTATT	pAEP4	FLAG-BC
FlagBC3	5′-CTTCTTTTAATTAATTATTTTTCCTGAAGACCGAG		
BCCP5	5′-CTTCTTCATATGGATATTCGTAAGATTAAAAAA	pAEP3	BCCP-BC
BCCP3	5′-CTTCTTGGATCCACCTCCTTACTCGATGACGACCAGCGGCTC		
BC5	5′-CTTCTTGGATCCGATGCTGGATAAAATTGTTATTGCC	pAEP7	
BC3	5′-CTTCTTCTCGAGTTATTTTTCCTGAAGAACGAG		
FlagCTa5	5′-CTTCTTCCATGG**ACTACAAAGACGATGACGATAAAA**TGAGTCTGAATTTCCTTGAT	pMEP1	FLAG-CT
FlagCTb3	5′-CTTCTTGGATCCTCAGGCCTCAGGTTCCTGATC		

[a]The underlined nucleotides are restriction endonuclease cut sites. The bold nucleotides are FLAG tag coding sequences.

after BCCP is carboxylated by biotin carboxylase, it dissociates and undergoes diffusion until it binds to carboxyltransferase. Alternatively, the second theory suggests that all the components of acetyl-CoA carboxylase form a complex in which the flexible region of BCCP alternates between the active sites of biotin carboxylase and carboxyltransferase.[3] The latter mechanism involves extensive protein−protein interactions (PPI). Because PPI are well-known to play significant roles in the regulation of many cellular processes such as binding of hormones to cell surface receptors, signal transduction, and allosteric regulation of enzymes, PPI may also play a role in the regulation of acetyl-CoA carboxylase activity.[14,15]

Understanding the regulation of bacterial acetyl-CoA carboxylase activity has become more pressing because of recent findings. It was recently proposed that during the stationary phase when acetyl-CoA levels are low, carboxyltransferase binds to the mRNA encoding the α- and β-subunits of the enzyme, resulting in inhibition of both catalysis and translation of the mRNA. When acetyl-CoA levels are high, such as in the log phase of *E. coli* growth, acetyl-CoA competes with the mRNA encoding the α- and β-subunits of carboxyltransferase, thereby allowing catalysis and translation to continue.[16] Thus, carboxyltransferase acts as a "dimmer" switch to regulate its own enzymatic activity and gene expression at the level of translation.

This leaves the question of how the activity of biotin carboxylase is regulated when carboxyltransferase is bound to its mRNA. Most importantly, does biotin carboxylase continuously carboxylate biotin and hydrolyze ATP, or is the activity decreased when carboxyltransferase is sequestered away bound to its mRNA? In this report, we answered these questions by demonstrating that all of the components of acetyl-CoA carboxylase form a multiprotein complex in which the protein−protein interactions play an integral part in regulating the activity of the enzyme.

EXPERIMENTAL PROCEDURES

Reagents. Amicon ultracentrifugal filters (molecular mass cutoff of 10000) were from Millipore. M280 streptavidin Dynabeads were from Invitrogen. The Bradford protein assay reagent, Opti-4CN substrate kit, gel filtration standards, goat anti-mouse IgG (H+L)−HRP conjugate, and avidin−HRP conjugate were purchased from Bio-Rad. Expression vectors, Perfect Protein Marker, Nova Blue competent cells, and BL21(DE3) competent cells were obtained from Novagen. Kanamycin, streptomycin, IPTG, and PINKstain Protein Ladder were from Gold Biotechnology. Restriction endonucleases, T4 DNA ligase, and dNTPs were purchased from New England Biolabs. Pfu UltraII fusion was from Agilent. FLAG octapeptide was synthesized by Chi Scientific. BioTrace PVDF Membrane was from PALL Life Sciences. The His-tag antibody anti-His monoclonal antibody from mouse was purchased from GenScript. Casamino acids were from Lab Scientific. Deuterium oxide was from Acros. All primers (Table 1) were synthesized by Eurofins MWG Operon. The HiLoad 16/60 Superdex 200 prep-grade gel filtration column was from Amersham Biosciences, while HisTrap HP Ni Sepharose columns were from GE Healthcare. All other reagents were from Sigma-Aldrich.

The 2-aminooxazole dibenzylamide inhibitor of biotin carboxylase was synthesized according to the method of Mochalkin et al.[17] The carboxyltransferase inhibitor, andrimid, was a gift from Pfizer. To measure the deuterium kinetic isotope effect on acetyl-CoA carboxylase, d_3-acetyl-CoA was synthesized by a modification of the procedure of Simon and Shemin.[18] First, in a 25 mL flask, 40 mg of the trilithium salt of coenzyme A was dissolved in 3 mL of 1 M triethylammonium bicarbonate. Next, 5 g of d_6-acetic anhydride was added to the coenzyme A solution dropwise with mixing. Once all the d_6-acetic anhydride had been added, the mixture was left on ice for 45 min and mixed periodically. The reaction mixture was then allowed to warm to room temperature over the course of 1 h. Then 1 mL of water and 3 drops of glacial acetic acid were

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added to the solution. The mixture stood at room temperature overnight. The following day, the reaction mixture was lyophilized to dryness, and the powder was resuspended in water. The concentration of d_3-acetyl-CoA was determined spectrophotometrically at 260 nm using an extinction coefficient of 16800 M^{-1} cm^{-1} for coenzyme A.

Expression Plasmid Construction. The plasmid encoding biotin carboxylase with an N-terminal FLAG tag (FLAG-BC) was produced by amplifying the *accC* gene using primers FlagBC5 and FlagBC3 (Table 1) and pGLW1 as a template.[19] The primers allowed for the introduction of both an *Nde*I site and the sequence encoding the FLAG tag on the 5′ end of the gene along with a *Pac*I site at the 3′ end. The polymerase chain reaction (PCR) product was cut with *Nde*I and *Pac*I and ligated into pETDuet-1 cut with the same enzymes to create pAEP4.

The plasmid encoding carboxyltransferase with an N-terminal FLAG tag on the α-subunit (FLAG-CT) was constructed by amplifying the *accA* and *accD* genes using pCZB3 as a template. Plasmid pCZB3 contains the genes encoding the α- and β-subunits of carboxyltransferase in a mini-operon.[8] Primers FlagCTa5 and FlagCTb3 (Table 1) introduced both an *Nco*I site and the sequence encoding the FLAG tag on the 5′ end of the *accA* gene and a *Bam*HI site on the 3′ end of the *accD* gene. The PCR product was cut with *Nco*I and *Bam*HI and ligated into pCDFDuet-1 cut with the same enzymes to create pMEP1.

A mini-operon of the *accB* and *accC* genes was constructed to generate the biotin carboxyl carrier protein−biotin carboxylase complex (BCCP−BC). The BCCP5 and BCCP3 primers were used to amplify the BCCP gene (*accB*) using pYML11 as a template. Plasmid pYML11 contains the gene for *E. coli* BCCP in pGEM7 between the *Eco*RI and *Bam*HI cut sites. The primers introduced an *Nde*I site on the 5′ end of the gene, and both the 5′-AAGGAG-3′ sequence, which encoded a ribosomal binding site, and a *Bam*HI site were added to the 3′ end of the gene. The PCR product was cut with *Nde*I and *Bam*HI and ligated into pET28b(+) cut with the same enzymes to create pAEP3. The *accC* gene was amplified from pGLW1 using primers BC5 and BC3 to introduce a 5′ *Bam*HI site and a 3′ *Xho*I site. The resulting PCR product was cut with *Bam*HI and *Xho*I and ligated into pAEP3 cut with the same enzymes to create pAEP7. This yielded a plasmid with an *accBC* mini-operon containing a 13 bp intercistronic region that expressed an N-terminally His-tagged BCCP and biotin carboxylase.

Protein Expression in *E. coli*. Biotin carboxylase with a His tag at the N-terminus[19] and carboxyltransferase with a His tag at the N-terminus of the α-subunit[8] were expressed and purified as previously described. Biotinylated BCCP with a His tag at the N-terminus was a gift from Pfizer.

FLAG-BC was produced by transforming *E. coli* strain BL21(DE3) with pAEP4 and then using a freshly transformed colony to inoculate either 0.5 L of LB medium in a 2 L flask or 1 L of LB medium in a 2.8 L Fernbach flask. The LB medium also contained 50 μg/mL ampicillin. The cells were incubated at 37 °C while being shaken until midlog phase. Then 250 μM IPTG was added to the culture, which was incubated for an additional 2.5−3.5 h while being cooled to 25 °C. The cells were harvested by centrifugation and frozen at −20 °C.

FLAG-CT was produced by transforming *E. coli* strain BL21(DE3) with pMEP1 and then using a freshly transformed colony to inoculate either 0.5 L of LB medium in a 2 L flask or 1 L of LB medium in a 2.8 L Fernbach flask containing either 10 mL glycerol or 1 g casamino acids. The LB medium also contained 50 μg/mL streptomycin. The flasks were incubated at 37 °C while being shaken until midlog phase and then allowed to stand and cool to room temperature. After the flasks had cooled, one of the following was added to induce expression of the gene encoding FLAG-CT: 250 μM IPTG, 1 g of lactose, or both 250 μM IPTG and 1 g of lactose. After the addition of IPTG or lactose, flasks were either incubated while being shaken for 2.5−3.5 h at room temperature or allowed to stand at room temperature overnight. The cells were harvested by centrifugation and frozen at −20 °C. A typical preparation would involve several flasks with different various combinations of inducing agents and incubation conditions.

Overexpression of holo-acetyl-CoA carboxylase was accomplished with a two-plasmid system in which plasmids pAEP7 and pMEP1 were both transformed into *E. coli* strain BL21(DE3). Overexpression was achieved with the same procedure as described above for FLAG-CT except that the LB medium contained 50 μg/mL streptomycin and 30 μg/mL kanamycin.

Protein Purification. The first step in protein purification was affinity chromatography using either a nickel-based resin for proteins with a His tag or an anti-FLAG M2 gel for proteins with a FLAG tag. For proteins with a His tag, the cell pellets were suspended in equilibration/wash buffer [20 mM NaH_2PO_4, 500 mM NaCl, and 15 mM imidazole (pH 7.8)] and then were lysed by sonication and passage through a French press. Nucleic acids were removed by the addition of DNase. The cell lysate was centrifuged at 34540*g* for 1 h at 4 °C. The supernatant was loaded onto a nickel affinity column pre-equilibrated with equilibration/wash buffer and then washed with 10 column volumes of the same buffer. Protein was eluted using a segmented gradient starting with 8 column volumes of 0−10% elution buffer [20 mM NaH_2PO_4, 500 mM NaCl, and 250 mM imidazole (pH 7.8)], followed by 12 column volumes of 10−100% elution buffer and finally 8 column volumes of 100% elution buffer. For proteins with a FLAG tag, the cell pellets were suspended in TBS wash [50 mM Tris-HCl and 150 mM NaCl (pH 7.4)] and lysed in the same manner as the His-tagged proteins. After centrifugation, the supernatant was loaded onto a column containing an anti-FLAG M2 affinity gel, and protein was eluted according to the protocol provided by the manufacturer (Sigma) of the anti-FLAG M2 affinity resin.

Protein from either the nickel affinity or anti-FLAG columns was further purified by size exclusion chromatography. Protein eluted from either affinity column was dialyzed for 12 h against a buffer that consisted of 134 mM KCl, 10 mM KH_2PO_4, and 1 mM EDTA (pH 7.8). The dialysate was then dialyzed for 12 h against 10 mM Hepes and 500 mM KCl (pH 7.8) (termed hereafter the second dialysis), concentrated using Amicon Centrifugal Filters, and loaded onto a Hi-Load 16/60 Superdex 200 column pre-equilibrated with second dialysis. Fractions containing pure protein were concentrated and stored at −80 °C.

Pull-Down Assays. Assays targeting biotinylated BCCP utilized M-280 streptavidin Dynabeads. A 100 μL suspension of beads was washed three times with 100 μL of PBS buffer [2.7 mM KCl, 10 mM Na_2HPO_4, and 1.76 mM KH_2PO_4 (pH 7.4)] as described in the manufacturer's protocol. Then 50 μL of 77.8 μM BCCP was added to the beads, and the beads were pelleted using a neodymium magnet. The beads were resuspended by being gently shaken at 25 °C for 1 h, pelleted again, and washed once with PBS buffer. A 450 μL solution of 1.67 mM

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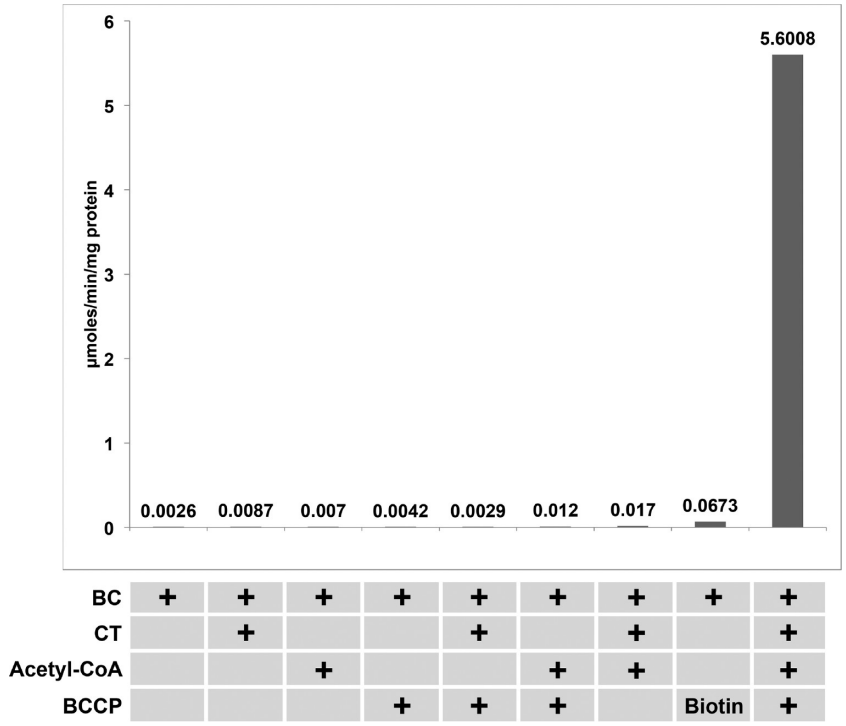

Figure 1. Effect of acetyl-CoA and the different components of acetyl-CoA carboxylase on the specific activity of biotin carboxylase. The specific activities were calculated on the basis of the amount (16.1 μg) of BC in each reaction mixture. Numbers above the bar indicate the specific activity; a plus sign below the bar indicates the presence of 0.32 μM BC, 0.24 μM CT, 2 μM BCCP, and/or 423 μM acetyl-CoA, and Biotin in the BCCP row indicates the presence of 8 mM biotin instead of BCCP. The concentrations of ATP and bicarbonate were held constant at 0.4 and 3.25 mM, respectively. All reactions were initiated by the addition of BC.

AMPPNP, 0.7 μM CT, 0.94 μM BC, 3.2 mM acetyl-CoA, 22.2 mM $MgCl_2$, 22.2 mM potassium bicarbonate, 111.2 mM Hepes (pH 7.8), and 22.2 mM biotin was added to the pelleted beads, which were resuspended by being mixed at 25 °C for 45 min. The beads were pelleted with a neodymium magnet, and the supernatant was removed. The pellet was washed four times with 200, 150, 100, and 90 μL of PBS, and 0.1% Tween 20 (PBST). After the last wash, the pellet was resuspended in 10 μL of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) buffer and 20 μL of PBST and incubated at 80 °C for 15 min. The beads were pelleted with a neodymium magnet, and the supernatant was removed for Western blot analysis.

For pull-down assays targeting FLAG-BC, anti-FLAG M2 affinity resin was washed three times with 190 μL of TBS. A solution of 55 μL of TBS and 55 μL of second dialysis containing 11.6 μM FLAG-BC was added to the anti-FLAG M2 resin and mixed at 25 °C for 1 h. After centrifugation, the supernatant was removed, and a 450 μL solution containing 1.7 mM AMPPNP, 0.9 μM CT, 6.9 μM BCCP, 3.2 mM acetyl-CoA, 22.2 mM $MgCl_2$, 22.2 mM potassium bicarbonate, and 111.2 mM Hepes (pH 7.8) was added and mixed at 25 °C for 1.5 h. The pellet was washed four times with 200, 150, 100, and 90 μL of TBS. After the last wash, the pellet was resuspended in 10 μL of SDS–PAGE buffer and 20 μL of 500 μg/mL FLAG peptide and incubated at 80 °C for 15 min. After centrifugation, the supernatant was removed for Western blot analysis. Pull-down assays targeting FLAG-CT were conducted exactly as described for the FLAG-BC pull-down assays with two exceptions. First, 55 μL of a 22 μM FLAG-CT solution was added to the washed resin instead of 55 μL of an 11.6 μM FLAG-BC solution. Second, the supernatant was removed after the sample had been mixed for 40 min, and a 445 μL solution of 1.7 mM AMPPNP, 0.95 μM BC, 4.0 μM BCCP, 3.3 mM acetyl-CoA, 22.5 mM $MgCl_2$, 22.5 mM potassium bicarbonate, and 112.5 mM Hepes (pH 7.8) was added and mixed at 25 °C for 1 h.

Western Blot Analysis. Samples were run on an SDS–PAGE gel and transferred to a PVDF membrane in transfer buffer (39 mM glycine, 48 mM Tris, 20% methanol, and 0.04% SDS) for 15 h at 60 V. The PVDF membrane with transferred PINKstain Protein Ladder was washed in TBST [136.9 mM NaCl, 2.68 mM KCl, 24.8 mM Tris (pH 7.4) with HCl, and 0.1% Tween 20] while being gently shaken at 25 °C for 20 min. The membrane was then blocked with 18 mL of TBST with 5% dry milk while being gently shaken at 25 °C for 1 h. Then a 1:3000 ratio of 1 μg/μL anti-His monoclonal antibody and anti-FLAG antibody from mouse was added to the TBST with 5% dry milk and incubated at 25 °C while being gently shaken for 1 h. The membrane was washed three times in 20 mL of TBST being gently shaken at 25 °C for 10 min each time and then incubated in 18 mL of TBST with 5% dry milk with a 1:4800 ratio of 1 μg/μL goat anti-mouse HRP conjugate antibody while being gently shaken at 25 °C for 1 h. After three more 10 min washes in TBST, the membrane was exposed to the Opti-4CN substrate for 15 min according to the instructions provided by the manufacturer (Bio-Rad).

Kinetic Assays. The activity of biotin carboxylase and holo-acetyl-CoA carboxylase was determined spectrophotometrically by measuring the production of ADP using pyruvate kinase and lactate dehydrogenase and following the oxidation of NADH at 340 nm. Each reaction mixture contained 17.5 units of lactate dehydrogenase, 10.5 units of pyruvate kinase, 0.5 mM phosphoenolpyruvate, 0.32 mM NADH, 20 mM $MgCl_2$, 15

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mM potassium bicarbonate, and 100 mM Hepes (pH 7.8). The activity of carboxyltransferase was measured as previously described.[a,8] All reactions were conducted in a total of 0.5 mL in a 1 cm path-length quartz cuvette. All reactions were initiated by addition of enzyme and mixtures maintained at 25 °C via a circulating water bath. Spectrophotometric data were collected using an Uvikon 810 spectrophotometer interfaced with a personal computer with a data acquisition program.

Kinetic Analysis. Data were analyzed by nonlinear regression using the computer programs of Cleland.[20] Initial velocities obtained by varying one of the substrates (A) were fit to eq 1 to determine the maximal velocity (V) and the Michaelis constant (K_m).

$$v = \frac{VA}{K_m + A} \quad (1)$$

When one substrate was varied at different fixed levels of another substrate, data were fit to eq 2 or 3, describing a sequential or ping-pong initial velocity pattern, respectively. In these equations, v is the experimentally determined velocity, V is the maximal velocity, K_a and K_b are the respective Michaelis constants for substrates A and B, respectively, and K_{ia} is the dissociation constant of A.

$$v = \frac{VAB}{K_{ia}K_b + K_aB + K_bA + AB} \quad (2)$$

$$v = \frac{VAB}{K_aB + K_bA + AB} \quad (3)$$

Data describing noncompetitive inhibition were fit to eq 4, where V is the maximal velocity, K_m is the Michaelis constant, I is the concentration of the inhibitor, and K_{is} and K_{ii} are the slope and intercept inhibition constants, respectively.

$$v = \frac{VA}{K_m\left(1 + \frac{I}{K_{is}}\right) + A\left(1 + \frac{I}{K_{ii}}\right)} \quad (4)$$

Multiple inhibition data were fit to eq 5, where K_L and K_J are the apparent dissociation constants for the two inhibitors and α is a measure of the degree of interaction of the two inhibitors.

$$v = \frac{V}{1 + \frac{L}{K_L} + \frac{J}{K_J} + \frac{LJ}{\alpha K_L K_J}} \quad (5)$$

RESULTS

Bacterial acetyl-CoA carboxylase, the *E. coli* form in particular, has been the model system for studying biotin-dependent enzymes for two reasons. First, the carboxylase and transferase components retain their activity when isolated, and second, both biotin carboxylase and carboxyltransferase will utilize free biotin (i.e., biotin not covalently attached to BCCP) as a substrate, thereby simplifying kinetic analysis.[13] While the use of free biotin has allowed for detailed kinetic and mechanistic studies of biotin carboxylase and carboxyltransferase, the fact remains that *in vivo* biotinylated BCCP, not free biotin, is the substrate for these two enzymes.[3] However, biotinylated BCCP turns out not to be a very good substrate for biotin carboxylase (Figure 1). In fact, the rate of ATP hydrolysis by biotin carboxylase is low even when all of the components of acetyl-CoA carboxylase are present (Figure 1). Not until acetyl-CoA is added is a dramatic increase in the rate of ATP hydrolysis detected (Figure 1). This phenomenon in which biotin carboxylase hydrolyzes ATP only in the presence of BCCP, carboxyltransferase, and acetyl-CoA was first noted by Soriano et al.[21] and Alves et al.[22] when these groups were developing high-throughput screening assays for bacterial acetyl-CoA carboxylase. The results in Figure 1 suggest there is communication between the active sites of biotin carboxylase and carboxyltransferase, and this by inference implies that all of the components form a higher-order protein complex. The active site communication is an example of substrate synergism whereby enzyme activity is regulated by allowing ATP to be utilized only when acetyl-CoA is present, thereby preventing wasteful hydrolysis of ATP.

Two distinct experimental approaches were used to address the active site communication and whether complex formation is necessary for the regulation of acetyl-CoA carboxylase activity. The first approach involved kinetic characterization of acetyl-CoA carboxylase, while the second approach used pull-down−co-immunoprecipitation assays to detect complex formation both *in vitro* and *in vivo*.

Kinetic Analysis. Any kinetic analyses of acetyl-CoA carboxylase must start by noting that the enzyme is considered to be a nonclassical or two-site ping-pong enzyme (Figure 2). If

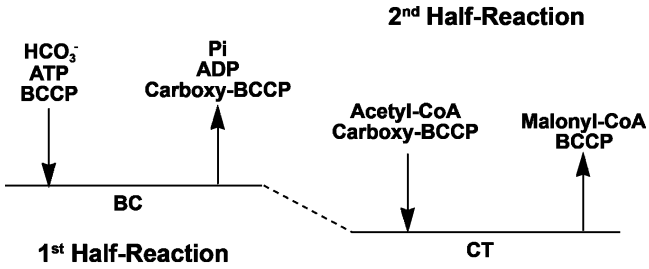

Figure 2. Kinetic mechanism of acetyl-CoA carboxylase as a two-site ping-pong mechanism.

acetyl-CoA carboxylase is indeed a two-site ping-pong (also known as double displacement) enzyme, then each of the two half-reactions is expected to exhibit activity in the absence of the substrate for the other half-reaction. In the case of acetyl-CoA carboxylase, one would expect ATP hydrolysis in the absence of acetyl-CoA. However, as has already been pointed out, that does not occur (Figure 1). Conversely, when carboxyltransferase is assayed in the nonphysiological direction with BCCP and malonyl-CoA as substrates, no activity could be detected in the absence of ATP (data not shown). This lack of reversibility is in stark contrast to the case for isolated carboxyltransferase, which is routinely assayed in the reverse direction.[a,8] Thus, the two half-reactions do not exhibit independent activity as expected of a ping-pong enzyme; in addition, the fact that the reaction is irreversible in acetyl-CoA carboxylase but not in isolated carboxyltransferase strongly suggests the individual components form a complex.

Further evidence that the two half-reactions are not separate is the fact that the K_m value for ATP in acetyl-CoA carboxylase is 1.7 μM, whereas in isolated biotin carboxylase, the K_m value is 80 μM.[19] This indicates the binding of ATP to biotin carboxylase is affected by the presence of BCCP and carboxyltransferase.

Isotope Effects. In the second half-reaction, the carboxyl group on carboxybiotin is transferred to acetyl-CoA. For the carboxylation reaction to occur, a proton must be removed

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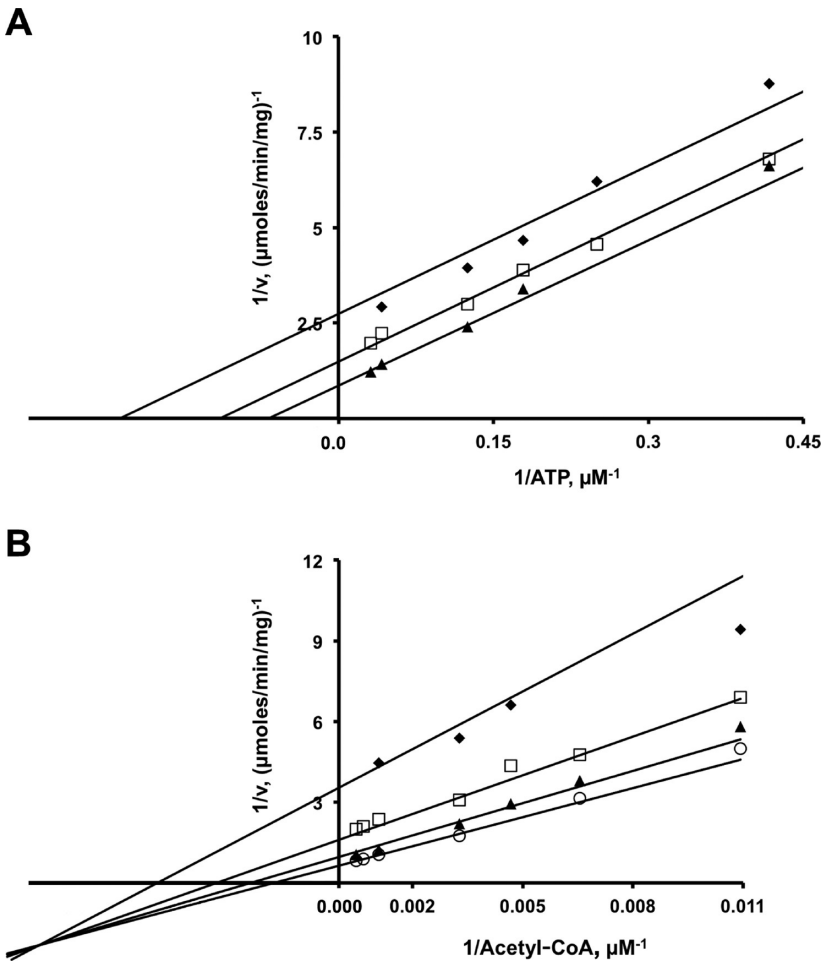

Figure 3. Initial velocity patterns of acetyl-CoA carboxylase. (A) Initial velocity pattern vs ATP concentration at different fixed levels of acetyl-CoA: 0.3 (◆), 0.6 (□), and 1.2 mM (▲). (B) Initial velocity pattern vs acetyl-CoA concentration at different fixed levels of ATP: 2 (◆), 5 (□), 10 (▲), and 20 μM (○). Both assays contained 2 μM BCCP, 0.32 μM BC, and 0.24 μM CT in each reaction mixture. The points represent the observed velocities, and the lines represent the best fits of the data to eq 3 (A) or 2 (B).

from the methyl group of acetyl-CoA. This allows the kinetic isotope effect for substituting deuterium for hydrogen in the methyl group of acetyl-CoA to be determined. If acetyl-CoA carboxylase has a conventional ping-pong kinetic mechanism, then the isotope effect when the ATP concentration is varied, $^{D}(V/K_{ATP})$, should be unity because the first half-reaction catalyzed by biotin carboxylase does not include the isotope sensitive step. $^{D}(V/K_{ATP})$ = 1.62 ± 0.01, while the isotope effect on the maximal velocity (^{D}V) is 1.8 ± 0.2. The lack of unity for $^{D}(V/K_{ATP})$ is inconsistent with a strict ping-pong mechanism and suggests an interaction between the two half-reactions.

The kinetic isotope effects also provide insight into the relative rates of the two half-reactions. For instance, the fact that the isotope effect on the maximal velocity (^{D}V) is not equal to the intrinsic isotope effect $(\sim 6.2)^{b}$ indicates that the second half-reaction catalyzed by carboxyltransferase is not completely rate-limiting. Moreover, because ^{D}V and $^{D}(V/K_{acetyl\text{-}CoA})$ are, within error, equal [$^{D}(V/K_{acetyl\text{-}CoA})$ = 2.1 ± 0.2], the first half-reaction catalyzed by biotin carboxylase is not completely rate-limiting; otherwise, ^{D}V would have been unity because the isotope sensitive step is part of the second half-reaction. Thus, the isotope effects suggest that both half-reactions are partly rate-limiting.

Initial Velocity Patterns. The initial velocity pattern for an enzyme with a ping-pong kinetic mechanism is expected to show a set of parallel lines in a double-reciprocal plot. In other words, when the concentration of the substrate for a particular half-reaction is varied, the slope in a double-reciprocal plot is not a function of the concentration of the substrate for the other half-reaction. When the ATP concentration was varied at different fixed levels of acetyl-CoA (Figure 3A), the data fit best to eq 3, which describes a set of parallel lines expected for a ping-pong kinetic mechanism.

In contrast, when the acetyl-CoA concentration was varied at different fixed levels of ATP (Figure 3B), a set of intersecting lines was observed. The data in Figure 3B were fit to both eqs 2 and 3. The data fit best to eq 2, describing a sequential initial velocity pattern, based on the fact that the average least squares of the residuals were the lowest for eq 2. The intersecting initial velocity pattern in Figure 3B indicates that all substrates (in this case ATP and acetyl-CoA) must be on the enzyme for catalysis to occur. A change in slope with an increasing ATP concentration means that even at very low acetyl-CoA concentrations, ATP affects the reaction rate. This suggests communication between the biotin carboxylase and carboxyltransferase active sites.

The two initial velocity patterns are seemingly contradictory in that when the ATP is varied at fixed levels of acetyl-CoA, a

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ping-pong mechanism is indicated, while the intersecting pattern when acetyl-CoA is varied at fixed levels of ATP is characteristic of a sequential mechanism. These two contradictory observations can be reconciled by noting that an initial velocity pattern for a sequential mechanism can resemble the pattern for a ping-pong mechanism. If the K_{ia} parameter in eq 2 (which is the dissociation constant for A) was much lower than the Michaelis constant for A (K_a), then eq 2 would be approximately equal to eq 3 and the initial velocity would appear as a set of parallel lines.[12] However, despite the appearance of a ping-pong kinetic mechanism, the true mechanism is in fact sequential.

It is important to point out that the kinetics observed here with the bacterial form of the enzyme are very similar to those observed with various forms of eukaryotic acetyl-CoA carboxylase. For instance, initial velocity patterns of acetyl-CoA carboxylase from chicken liver,[24] maize,[25] and the human mitochondrial isoform[26] all exhibit an intersecting initial velocity pattern when acetyl-CoA is varied at different fixed levels of ATP and when ATP is varied at different fixed levels of acetyl-CoA. The authors of each of those studies concluded that the two different active sites in the various eukaryotic acetyl-CoA carboxylases interact or communicate with each other and that the enzyme does not have a strict ping-pong kinetic mechanism.

Inhibition Studies. Given that acetyl-CoA carboxylase did not show an initial velocity pattern expected of a ping-pong enzyme, dead-end inhibition analyses were conducted to further probe the kinetic properties of the enzyme. Enzymes with ping-pong kinetic mechanisms would be expected to exhibit uncompetitive inhibition when the substrate for a half-reaction is varied with different fixed levels of an inhibitor that binds to the enzyme catalyzing the other half-reaction. Therefore, an inhibitor of biotin carboxylase was examined as a function of varying levels of acetyl-CoA, while an inhibitor of carboxyltransferase was examined as a function of varying levels of ATP.

The biotin carboxylase inhibitor, 2-aminooxazole dibenzylamide, was developed by Pfizer as an antibiotic and was found to be competitive with respect to ATP.[17] The carboxyltransferase inhibitor, andrimid, is a natural product antibiotic that exhibits competitive inhibition with respect to acetyl-CoA.[27] The 2-aminooxazole dibenzylamide inhibitor of biotin carboxylase would be expected to exhibit uncompetitive inhibition with respect to acetyl-CoA if acetyl-CoA carboxylase is a strictly ping-pong mechanism. Likewise, the carboxyltransferase inhibitor, andrimid, would be expected to exhibit uncompetitive inhibition with respect to ATP. However, as shown in panels A and B of Figure 4, 2-aminooxazole dibenzylamide is a noncompetitive inhibitor versus acetyl-CoA and andrimid is a noncompetitive inhibitor versus ATP, respectively. The noncompetitive inhibition pattern indicates that inhibitor binding at the active site of either biotin carboxylase or carboxyltransferase is affecting binding and catalysis in the other enzyme, which again implies communication between the two proteins.

Multiple Inhibition. Because inhibitor binding at one active site affected the activity of the other enzyme, multiple-inhibition analysis was undertaken to determine whether binding of the inhibitor to the active sites of biotin carboxylase and carboxyltransferase was synergistic or antagonistic. Multiple inhibition analyses were conducted by measuring the initial velocities at increasing concentrations of one inhibitor while the second inhibitor was held constant. Initial velocities were

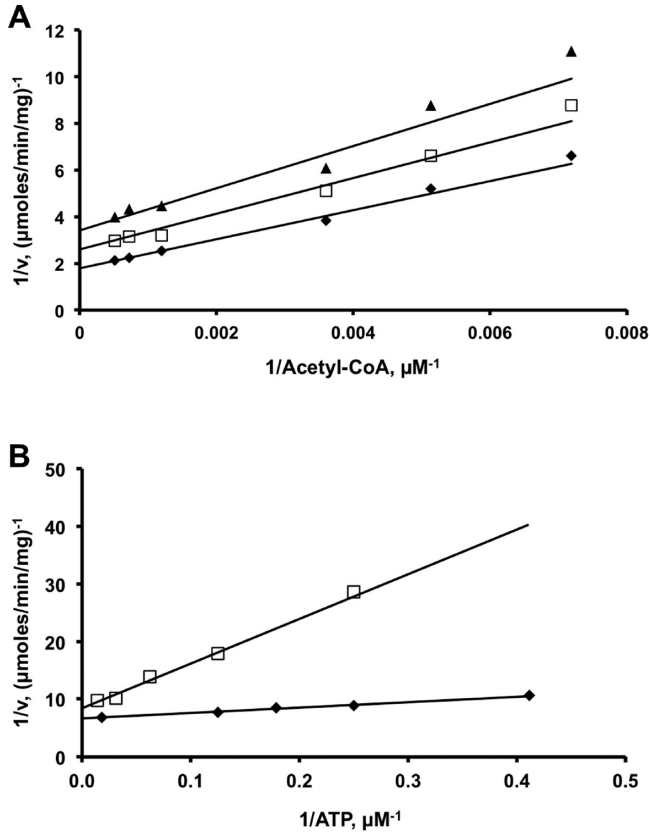

Figure 4. Dead-end inhibition of acetyl-CoA carboxylase. (A) Inhibition by 2-aminooxazole dibenzylamide at 0 (◆), 0.5 (□), and 1 μM (▲) while varying acetyl-CoA and holding the ATP concentration constant at 10 μM. (B) Inhibition by andrimid at 0 (◆) and 15 nM (□) while varying ATP and holding the acetyl-CoA concentration constant at 200 μM. Both assays contained 2 μM BCCP, 0.32 μM BC, and 0.24 μM CT in each reaction mixture. The points represent the observed velocities, and the lines represent the best fit of the data to eq 4.

measured again at higher levels of the second inhibitor and then plotted as the inverse velocity versus the concentration of the first inhibitor (sometimes termed a Yonetani–Theorell plot).[28] The substrate concentrations were held constant at subsaturating levels. For multiple-inhibition analysis of acetyl-CoA carboxylase, one inhibitor was the biotin carboxylase inhibitor 2-aminooxazole dibenzylamide. For the carboxyltransferase inhibitor, the product malonyl-CoA was used instead of andrimid because malonyl-CoA is water-soluble whereas andrimid is dissolved in DMSO. The levels of andrimid required for multiple-inhibition studies would exceed the amount of DMSO that acetyl-CoA carboxylase can tolerate.

When 2-aminooxazole dibenzylamide was varied at different fixed levels of malonyl-CoA, an intersecting pattern was observed that indicates the two inhibitors can bind to the enzyme simultaneously (Figure 5). When the data were fit to eq 5, an α value of 0.69 was obtained. The α value is an indication of the interaction of the two inhibitors. α values of >1 indicate that the bindings of the two inhibitors interfere with one another; an α value of 1 indicates no interaction between the inhibitors, and an α value of <1 indicates synergism in the binding of the inhibitors. Thus, the fact that two inhibitors, which bind to biotin carboxylase and carboxyltransferase, interact synergistically suggests an interaction between the active sites.

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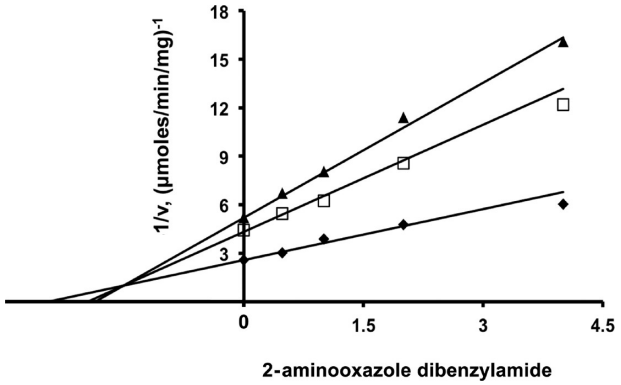

Figure 5. Multiple inhibition of acetyl-CoA carboxylase. Inhibition varying 2-aminooxazole dibenzylamide at different fixed levels of malonyl-CoA: 0 (◆), 200 (□), and 300 μM (▲). Concentrations of ATP, acetyl-CoA, BCCP, BC, and CT were held constant at 10, 399, 2, 0.32, and 0.24 μM, respectively. The points represent the observed velocities, and the lines represent the best fit of the data to eq 5.

In summary, the steady-state kinetic analysis of acetyl-CoA carboxylase taken as a whole indicates the enzyme undergoes a sequential kinetic mechanism. For example, all the substrates must be on the enzyme for catalysis to occur. The fact that acetyl-CoA carboxylase does not behave as a ping-pong enzyme and the fact that the two-half reactions are interacting imply a physical association between the proteins. To measure directly if the proteins comprising the multifunctional acetyl-CoA carboxylase form a complex, pull-down or co-immunoprecipitation assays were employed.

Pull-Down Assays. The rationale behind the pull-down assays was simply to pull down one of the components of acetyl-CoA carboxylase and see if the other two components are also pulled down. Biotin carboxylase or carboxyltransferase was targeted for pull-down or co-immunoprecipitation assay via the placement of an octapeptide FLAG tag on the N-terminus of the protein. This allows anti-FLAG binding resin (which is an antibody to the FLAG peptide) to be used to pull down any complexes that form. To target biotinylated BCCP, the protein streptavidin was used because it binds biotin very tightly (K_d = 10^{-15} M).[29]

Pull-down assays of BCCP with streptavidin in the presence of carboxyltransferase and biotin carboxylase showed that all three components do indeed form a complex and that when BCCP was removed, only slight nonspecific binding by carboxyltransferase was observed (Figure 6, lanes 5 and 6). Likewise, when FLAG-CT was pulled down in the presence of biotin carboxylase and BCCP, all three proteins were detected, and when FLAG-CT was removed, neither biotin carboxylase nor BCCP was observed (Figure 6, lanes 7 and 8). Finally, both carboxyltransferase and BCCP were pulled down when FLAG-BC was targeted and were not pulled down when FLAG-BC was absent (Figure 6, lanes 9 and 10). Thus, irrespective of which component of acetyl-CoA carboxylase is targeted, the other two components are also pulled down. These data strongly suggest these proteins can form a complex, at least *in vitro*, and are consistent with the steady-state kinetic analysis.

To determine if the substrates have an effect on complex formation, pull-down assays targeting FLAG-BC were conducted in the presence or absence of acetyl-CoA and the nonhydrolyzable analogue of ATP, AMPPNP. As shown in Figure 7, neither acetyl-CoA nor AMPPNP had any effect on

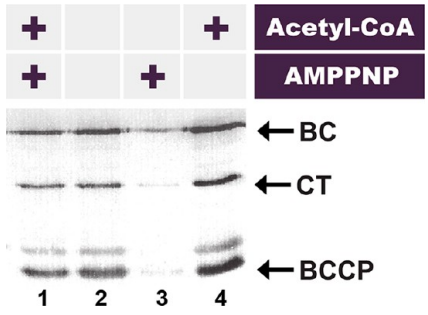

Figure 7. Effect of acetyl-CoA and AMPPNP on the pull-down assays targeting the individual components of acetyl-CoA carboxylase. In all lanes, FLAG-BC was targeted with anti-FLAG resin to pull down CT and BCCP: lane 1, pull-down assay in the presence of 3.22 mM acetyl-CoA and 1.67 mM AMPPNP; lane 2, pull-down assay in the absence of acetyl-CoA and AMPPNP; lane 3, pull-down assay in the absence of acetyl-CoA and in the presence of 1.67 mM AMPPNP; lane 4, pull-down assay in the presence of 3.22 mM acetyl-CoA and in the absence of AMPPNP.

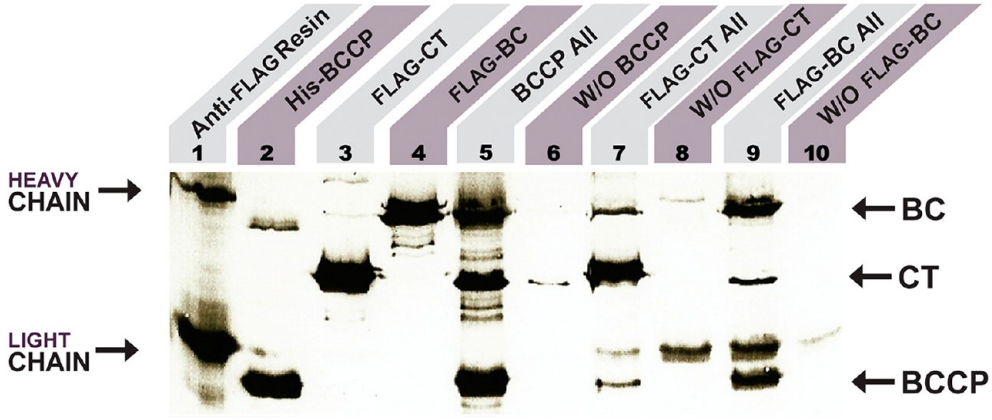

Figure 6. Pull-down assays targeting the individual components of acetyl-CoA carboxylase: lane 1, anti-FLAG resin showing the heavy and light chains of the anti-FLAG resin antibodies; lanes 2–4, input lanes of His-BCCP, FLAG-CT, and FLAG-BC, respectively; lanes 5 and 6, pull-down assay using streptavidin beads to target biotinylated BCCP in the presence of BC and CT and in the absence of BCCP, respectively; lanes 7 and 8, pull-down assay using anti-FLAG resin to target FLAG-CT in the presence of BCCP and BC and in the absence of FLAG-CT, respectively; lanes 9 and 10, pull-down assay using anti-FLAG resin to target FLAG-BC in the presence of BCCP and CT and in the absence of FLAG-BC, respectively.

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complex formation. The lane containing only AMPPNP has similar band intensities within the lane, and the reduction in band intensities in comparison to those of the other lanes is due to a reduced amount of resin at the end of the experiment. In addition, the mRNA encoding the α-subunit of carboxyltransferase, which has been shown to bind to carboxyltransferase,[16] also did not have any effect on complex formation (data not shown).

While the pull-down assays showed the three components of acetyl-CoA carboxylase form a complex *in vitro*, the next question was whether the multiprotein complex could also form *in vivo*. Initially, the bacterial two-hybrid system described by Dove et al.[30] was used to detect *in vivo* interactions. This approach proved to be problematic and inconclusive, most likely because the technique involved fusing proteins to the N-termini of biotin carboxylase and carboxyltransferase. Therefore, to examine complex formation *in vivo*, a mini-operon containing the genes for the α- and β-subunits of carboxyltransferase was constructed in one plasmid, while another plasmid with a different origin of replication contained a mini-operon with the genes encoding biotin carboxylase and an N-terminally His-tagged BCCP. Overexpression of the genes, cell lysis, and purification of the His-tagged BCCP by nickel affinity chromatography revealed that all of the subunits of acetyl-CoA carboxylase are copurified as a single complex (Figure 8). The

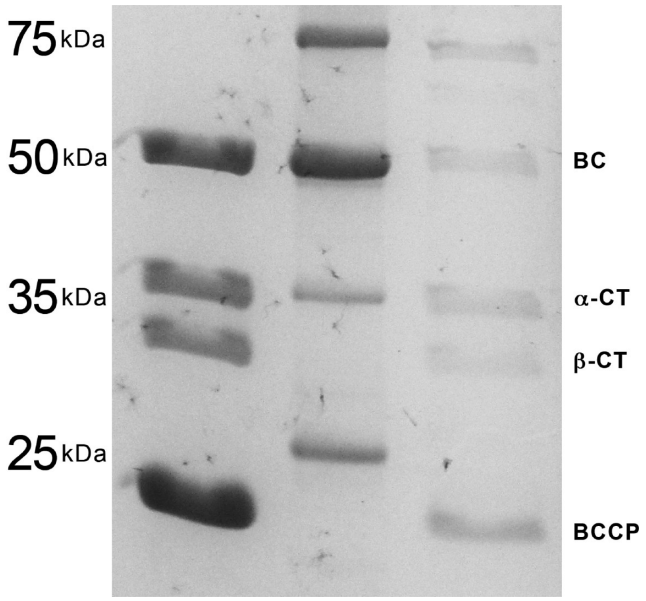

Figure 8. Pull-down assay of acetyl-CoA carboxylase produced *in vivo*: lane 1, SDS–PAGE of ACC after purification on a nickel affinity column; lane 2, molecular mass markers; lane 3, SDS–PAGE of ACC after affinity purification on an anti-FLAG column.

complex remained intact even after gel filtration chromatography, where the major peak of acetyl-CoA carboxylase activity eluted before the 640 kDa standard (Figure 9A,B). A molecular mass of >640 kDa indicates that the multiprotein complex is not simply composed of a biotin carboxylase homodimer bound to two monomers of BCCP and a heterotetramer of carboxyltransferase that would have a molecular mass of 269 kDa. Instead, the data suggest acetyl-CoA carboxylase is a large macromolecular complex. The stoichiometry of the multiprotein complex is not known and will have to await the determination of the crystal structure of acetyl-CoA carboxylase, which is underway.

To determine if *in vivo* complex formation is not simply due to using His-tagged BCCP, the whole process was repeated using FLAG-CT as the targeted protein and anti-FLAG resin in place of the nickel affinity resin. The same results were obtained in that all three components were copurified on the anti-FLAG affinity column (Figure 8). To determine if the acetyl-CoA carboxylase complex is only stable when it is formed *in vivo*, purified biotin carboxylase, BCCP, and carboxyltransferase were combined and subjected to gel filtration chromatography. Just like the acetyl-CoA carboxylase that was produced *in vivo*, a peak eluting before the 640 kDa standard was observed (Figure 9A). SDS–PAGE analysis of the major peak showed it contained the four separate proteins that comprise acetyl-CoA carboxylase (Figure 10). The bands corresponding to the α- and β-subunits of CT in Figure 10 are slightly different from the *in vivo*-assembled acetyl-CoA carboxylase. Because the stoichiometry of the multiprotein complex is unknown, excess BCCP and BC were mixed with CT to ensure complex formation. Excess BCCP-BC forms a complex with a >640 kDa quaternary structure[31] that is outside the resolution range of the size exclusion column that would allow separation from holo-ACC and accounts for the differing band intensities in Figure 10.

Thus, all of the evidence taken together (enzyme kinetics along with the *in vitro* and *in vivo* pull-down assays) strongly argues that acetyl-CoA carboxylase forms a higher-order protein structure and that formation of this multiprotein complex is necessary for regulating the enzymatic activity whereby ATP is hydrolyzed only in the presence of acetyl-CoA.

DISCUSSION

Protein–protein interactions play a multitude of roles in cellular and organismal functions such as signal transduction, blood coagulation, immunity, etc. The complexes involved in PPI have been characterized as being either obligate or nonobligate.[14,32] Obligate PPI are distinguished from nonobligate PPI in that the different protein components cannot exist independently and that the protein interactions are irreversible.[15] Acetyl-CoA carboxylase seems to fall into both categories because biotin carboxylase and carboxyltransferase can be isolated and retain their enzymatic function indicative of a nonobligate PPI. However, all the components together form a multimeric complex that appears to be stable, at least to the extent that it remains intact after size exclusion chromatography, consistent with an obligate PPI. The results in this study provide the first evidence that all the components of acetyl-CoA carboxylase form a complex, which is in direct contrast to previous reports that the multienzyme complex is labile and transient in nature.[3,33]

These seemingly contrasting observations can be reconciled by noting that while biotin carboxylase and carboxyltransferase do indeed retain their catalytic activity when isolated, that activity is only observed when free biotin is used as a substrate.[13] When biotin covalently linked to BCCP is the substrate, biotin carboxylase or carboxyltransferase has little to no activity. It is only when acetyl-CoA and all three components, biotin carboxylase, BCCP, and carboxyltransferase, are present that the enzyme exhibits full enzymatic activity. Therefore, the PPI in acetyl-CoA carboxylase could be termed obligate because the individual components do not function independently and the interactions are irreversible. The

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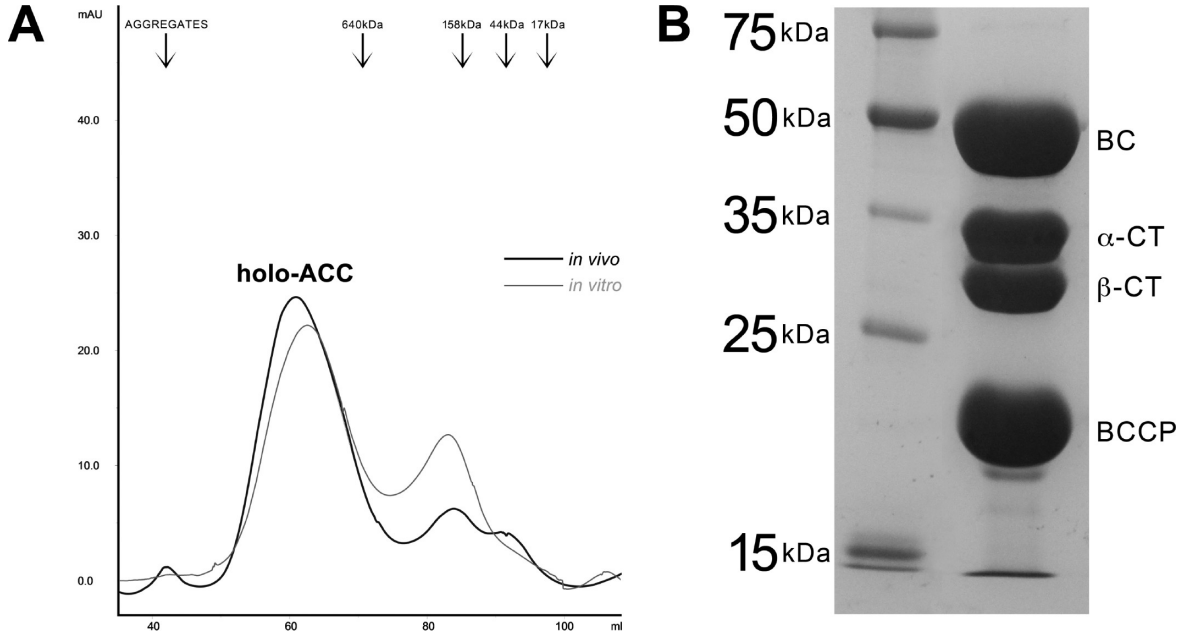

Figure 9. Gel filtration of acetyl-CoA carboxylase produced *in vivo* and *in vitro*. (A) The solid black and gray lines denote the mAU of the elution off the size exclusion column for *in vivo* and *in vitro* holo-ACC, respectively, and holo-ACC marks the peak containing ACC from the *in vivo* run, which corresponds to lane 2 in panel B. The arrows at the top of the figure denote the milliliters at which the molecular mass markers elute. (B) Lane 1 contained molecular mass markers and lane 2 *in vivo* ACC after purification on a nickel affinity column followed by a size exclusion column.

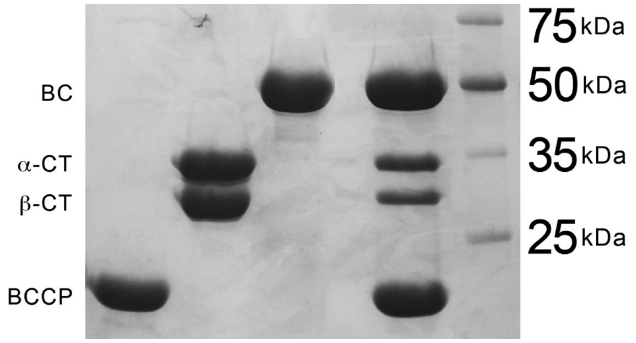

Figure 10. SDS−PAGE of acetyl-CoA carboxylase produced *in vitro*. Separately purified BC, BCCP, and CT were combined *in vitro* and then subjected to size exclusion chromatography: lane 1, BCCP; lane 2, FLAG-CT; lane 3, BC; lane 4, *in vitro* ACC after combining lanes 1−3 and purification over a size exclusion column; lane 5, molecular mass markers.

question now is why isolated biotin carboxylase and carboxyltransferase have activity with free biotin but not with biotin covalently linked to BCCP.

The N-terminal domain of BCCP appears to be the structural feature that impedes the enzymatic activity of isolated biotin carboxylase or carboxyltransferase when BCCP is the substrate. This conclusion is based on the observation that the biotinylated, C-terminal domain of BCCP (termed BCCP87 because it contains 87 amino acids) was an excellent substrate for isolated biotin carboxylase and carboxyltransferase.[34] In fact, the catalytic efficiencies of BCCP87 for isolated biotin carboxylase and carboxyltransferase were 8000- and 2000-fold higher than that for free biotin, respectively.[35] In other words, in the absence of the N-terminal domain, BCCP works quite well as a substrate for either biotin carboxylase or carboxyltransferase. Therefore, one possible role for the N-terminal domain of BCCP is to inhibit catalysis by biotin carboxylase, thereby preventing wasteful ATP hydrolysis in the absence of carboxyltransferase and/or acetyl-CoA.

The lack of reversibility in the reaction catalyzed by acetyl-CoA carboxylase actually supports the idea that acetyl-CoA carboxylase catalyzes the rate-determining step in fatty acid synthesis.[36] Specifically, the fact that acetyl-CoA carboxylase will not catalyze the reverse reaction (i.e., the conversion of malonyl-CoA into acetyl-CoA) means the first step in fatty acid synthesis is not at equilibrium, which is exactly what is wanted in a reaction that is the rate-determining step in a metabolic pathway. The consequence of the irreversibility of acetyl-CoA carboxylase is that all of the product of the reaction, malonyl-CoA, will be utilized by fatty acid synthase, and thus, the flux through fatty acid synthesis will be at least partly determined by the number of enzyme molecules. This concept fits perfectly with the model proposed by Meades et al.,[16] where, in the stationary phase of *E. coli* growth, carboxyltransferase acts as a "dimmer switch" by binding to the mRNA encoding the α- and β-subunits, which inhibits translation as well as enzymatic activity. In contrast, during the log phase, acetyl-CoA levels increase dramatically and compete with mRNA for binding to carboxyltransferase. This allows for substrate turnover as well as translation of mRNA to synthesize more carboxyltransferase molecules, resulting in an increase in flux through fatty acid synthesis. Thus, acetyl-CoA, which acts as a sensor of the metabolic state of *E. coli*, regulates acetyl-CoA carboxylase, first by upregulating the synthesis of the carboxyltransferase component and second by stimulating enzymatic activity. These two levels of regulation mediated by acetyl-CoA minimize the unnecessary hydrolysis of ATP when acetyl-CoA levels are low, while at the same time allowing the flux of the fatty acid synthetic pathway to be controlled by the number of enzyme molecules.

Lastly, the work described here not only examines the basic mechanism and regulation of acetyl-CoA carboxylase but also has potential medical ramifications. Both biotin carboxylase and

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carboxyltransferase have been validated as new targets for antibiotic development. Compounds that inhibit biotin carboxylase[17,37] and carboxyltransferase[27] have been found to have antibacterial activity. These compounds inhibit biotin carboxylase and carboxyltransferase in the traditional manner, by binding in the active sites and preventing substrate binding. However, because acetyl-CoA carboxylase clearly functions as a multiprotein complex, this greatly expands the number of potential target sites beyond only the active sites to also include the sites of protein−protein interactions for the different components of acetyl-CoA carboxylase. Moreover, the multienzyme complex provides the opportunity to make a multiligand inhibitor that incorporates both biotin carboxylase and carboxyltransferase inhibitors. Not only would an acetyl-CoA carboxylase multiligand inhibitor be potentially more potent than either of the individual ligands, but a multiligand antibacterial agent would also lower the likelihood of pathogenic bacteria developing resistance.[38−41]

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Notes

The authors declare no competing financial interest.

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DEDICATION

This paper is dedicated to the memory of Professor W. W. Cleland.

ABBREVIATIONS

BCCP, biotin carboxyl carrier protein; FAS II, fatty acid synthesis type II; FAS I, fatty acid synthesis type I; ACC, acetyl-CoA carboxylase; BC, biotin carboxylase; CT, carboxyltransferase; PPI, protein−protein interactions; FLAG-BC, biotin carboxylase with an N-terminal FLAG tag; FLAG-CT, carboxyltransferase with an N-terminal FLAG tag on the α-subunit; BCCP−BC, biotin carboxyl carrier protein−biotin carboxylase.

ADDITIONAL NOTES

[a]The carboxyltransferase subunit from *E. coli* is routinely assayed in the nonphysiological direction with an assay that couples the production of acetyl-CoA with the reduction of NAD^+ by the combined reactions of citrate synthase and malate dehydrogenase.[8] This assay utilizes biocytin in place of biotin because biocytin gives 3-fold higher maximal velocities than does biotin.[8] Biocytin is biotin with a lysine linked to the valeric acid side chain via an amide bond with the ϵ-amino group.

[b]The removal of a proton from the methyl group of acetyl-CoA is analogous to a keto−enol conversion. Keeffe and Kresge measured a deuterium isotope effect on the enolization of isobutyrophenone of 6.2.[23] Therefore, the intrinsic isotope effect for substituting deuterium for hydrogen at the methyl group of acetyl-CoA is estimated to be ∼6.2.

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