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Consequences of Reduced Intracellular Coenzyme A Content in *Escherichia coli*

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***Escherichia coli* β-alanine auxotrophs (*panD2*) were used to manipulate the specific cellular content of coenzyme A (CoA) and assess the associated physiological effects. Growth-limiting concentrations of CoA resulted in an increase in phospholipid/protein ratio in *relA1* mutants, but not in their *rel*$^+$ counterparts, indicating that protein biosynthesis was more severely affected by CoA deprivation than phospholipid biosynthesis. Acetyl-CoA was the dominant component (79.8%) of the CoA pool in cells exponentially growing in glucose-minimal medium, with significant concentrations of CoA (13.8%) and succinyl-CoA (5.9%) also detected. Malonyl-CoA was a minor species (0.5%), and the mixed disulfide of CoA and glutathione was not present. Acetyl-CoA was also the major constituent in cells depleted of CoA. On the other hand, succinyl-CoA was absent, suggesting that the protein synthesis defect may be due to the inability to generate sufficient quantities of precursors via the tricarboxylic acid cycle to support amino acid biosynthesis. Production of acyl carrier protein was controlled in part by the availability of CoA, and the lower concentration of acyl carrier protein in CoA-depleted cells was associated with a concomitant decrease in the saturated/unsaturated fatty acid ratio.**

Coenzyme A (CoA) and acyl carrier protein (ACP) are the major cellular constituents in *Escherichia coli* that are biosynthesized from the vitamin pantothenic acid (1, 22). Both compounds function as coenzymes in the metabolism of acyl moieties which are bound as thioesters to the terminal sulfhydryl of the 4′-phosphopantetheine portion of these molecules. These two cofactors are interrelated, in that CoA serves as the donor of 4′-phosphopantetheine to apo-ACP (8), and the prosthetic group of ACP turns over (13, 21), thus releasing 4′-phosphopantetheine that is either reused for CoA biosynthesis (14, 21) or irreversibly excreted from the cell (14). Several investigators have observed that the intracellular concentration of CoA can be manipulated by varying the amount of pantothenate (1, 11) or β-alanine (11, 12, 14, 21) in the growth medium of mutant strains that are unable to synthesize pantothenate or one of its precursors. The observation that growth stasis ensues when the concentration of CoA falls below 5 pmol/10^8 cells (12) illustrates the importance of CoA to cell growth, although a more specific description of the metabolic consequences of an insufficient cellular CoA supply is not available. Interference with phospholipid production appears to be a likely possibility, since both CoA and ACP function as cofactors in fatty acid metabolism. However, CoA thioesters are directly used in the biosynthesis of five amino acids and are indirectly required for macromolecular biosynthesis by the participation of acetyl-CoA in the synthesis of glutamate via the tricarboxylic acid cycle (10). The goal of the present work was to determine whether protein or phospholipid production is most sensitive to depletion of CoA.

MATERIALS AND METHODS

Materials. Sources of supplies were Amersham Corp. (Arlington Heights, Ill.), β-[3-^{3}H]alanine (specific activity, 40 or 34 Ci/mmol) and ACS scintillation solution; ICN Pharmaceuticals, Inc. (Irvine, Calif.), [1-^{14}C]acetyl-CoA (specific activity, 0.056 or 0.0441 Ci/mmol); New England Nuclear Corp. (Boston, Mass.), [2-^{14}C]malonyl-CoA (specific activity, 0.0469 Ci/mmol); Serdary Research Laboratories, phosphatidylethanolamine and other lipid standards; Analtech, 250-μm Silica Gel H plates; Whatman, Inc. (Clifton, N.J.), DEAE-cellulose (DE-52); Anspec, Spherisorb ODS II chromatography column; Sigma Chemical Co. (St. Louis, Mo.), lysozyme, Tris, dithiothreitol, β-alanine, the mixed disulfide of CoA and glutathione (CoASSG), and bovine serum albumin; P-L Biochemicals Inc. (Milwaukee, Wis.), acetyl-CoA, malonyl-CoA, acetoacetyl-CoA, and succinyl-CoA; and Supelco Inc., DEGS-PS gas chromatography column. [*pan*-^{3}H]ACP and [*pan*-^{3}H]CoA standards were prepared biosynthetically with strain SJ16 and purified by DEAE-cellulose chromatography (11). All other chemicals and solvents were reagent grade or better.

Bacterial strains. The strains used in this study were strain SJ16 (*panD2 zad-220*::Tn*10 relA1 metB1 spoT1 gyrA216* λ^- λ^r F^-) (11), strain SJ30 (*panD2 zad-220*::Tn*10 metB1 argA52 spoT1* λ^- λ^r F^-), and strain SJ31 (*panD2 zad-220*::Tn*10 relA1 metB1 argA52 spoT1* λ^- λ^r F^-). Strains SJ30 and SJ31 were derived from strains NF161 and NF162 (9), respectively, by P1-mediated transduction with phage stocks grown on strain SJ16. Strains that harbor the *panD2* (5) mutation lack aspartate-1-decarboxylase activity (4, 29) and have an absolute growth requirement for β-alanine.

Growth conditions. Strains were grown on minimal medium E (27), supplemented with glucose (0.4%), thiamine (0.001%), required amino acids (0.01%), and the indicated amount of β-alanine. Cells from overnight cultures were washed and used to inoculate the experimental cultures at a density of 2×10^6 cells per ml. Cultures were incubated at 37°C with shaking. β-[3-^{3}H]alanine (specific activity, 40 Ci/mmol) was the sole source of β-alanine at concentrations

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of 1 μM or less. At higher β-alanine concentrations, unlabeled β-alanine was added to 1 μM β-[3-^{3}H]alanine to increase the final concentration in the medium, and the overall specific activity of the β-[3-^{3}H]alanine was reduced accordingly. When *panD* strains were grown on a range of β-alanine concentrations, it was first necessary to deplete the endogenous intracellular CoA pool before incubation in the desired amount of β-alanine (11). Media containing the indicated concentrations of β-alanine were then inoculated with the CoA-depleted cells to a density of 2×10^6 cells per ml, and the cultures were incubated for 14 h at 37°C and harvested. Harvested cells were washed twice with growth medium at 0 to 4°C and stored overnight at −70°C.

Analytical techniques. Cell extracts were prepared essentially as described by Showe and DeMoss (24). Cells were washed once with 50 mM Tris hydrochloride, pH 7.5, and 1 mM $MgCl_2$ and then resuspended in 0.4 ml of the same buffer containing 100 μg of lysozyme per ml and 4 μg DNase I per ml. The suspension was freeze-thawed three times with a dry ice and ethanol bath and a 37°C water bath, followed by sonication in a cuphorn attached to a Heat Systems sonicator for three 10-s intervals at an output setting of 10. The resulting sample was then centrifuged for 15 min in a Beckman Microfuge-12 (Beckman Instruments, Inc., Fullerton, Calif.). The pellet contained less than 2% of the ACP plus CoA in the total extract. The amount of CoA and ACP in the β-alanine-labeled cell extracts was determined by thin-layer chromatography (11).

Protein was determined by using the microbiuret method (20), with bovine serum albumin as the standard. Cell pellets were dissolved in 1 N NaOH at 60°C prior to protein measurements. Phospholipids were extracted from 1 or 2 ml of cell cultures by the method of Bligh and Dyer (2) and quantitated colorimetrically by the procedure of Stewart (25), with *E. coli* phosphatidylethanolamine as the standard. Fatty acid methyl esters were prepared by reacting the lipid extract with 5% HCl in anhydrous methanol. Methyl esters were extracted into hexane, dissolved in carbon disulfide, and the fatty acid compositions were determined using a Varian 3700 gas chromatograph equipped with a column packed with Supelcoport (100/120 mesh) coated with 5% DEGS-PS and operated isothermally at 165°C. Integration accuracy was checked by using the NHI-D standard mixture.

Cell number was determined by turbidity measurements, using a Klett-Summerson colorimeter with a blue filter. The colorimeter was calibrated by determining CFU per milliliter at points corresponding to the range of readings encountered. The calibration was performed repeatedly with strain SJ16, and the results were in close agreement with those obtained by using the unrelated laboratory strain C600. The specific cellular contents of CoA, phospholipid, protein, and ACP were calculated from the analytical determinations and the turbidity measurements. The intracellular concentrations of CoA species were calculated with 0.437 μl/5×10^8 cells as the cell volume (26, 30).

Quantitation of CoA thioesters. Strain SJ16 was labeled with β-[3-^{3}H]alanine as described above, and the cells from 1 ml of culture were harvested by a 10-min centrifugation in a Beckman Microfuge-12 maintained at 4°C. The pellets were washed twice with cold (0 to 4°C) growth medium and were then suspended in 25 μl of 1 N formic acid and vortex mixed for 15 s every 5 min for a total of 30 min. During the extraction procedure the samples were held in an ice slush and then stored at −70°C. The composition of the CoA pool did not change upon storage (up to 1 week). Just prior to analysis, 25 μl of 1 M dibasic potassium phosphate was added (final pH, 6.5), and the sample was filtered by using an Amicon micropartition system equipped with a YMT ultrafiltration membrane (Amicon Corp., Lexington, Mass.). This step removed >99% of the UV-absorbing material from the sample, and the recovery of radioactive CoA was 90% or greater. Radioactive ACP was partially retained (66%) by the membrane.

CoA species were fractionated by a modification of the method of DeBuysere and Olson (6). A Perkin-Elmer series 400 liquid chromatograph (The Perkin-Elmer Corp., Norwalk, Conn.) was equipped with a 5-μm Spherisorb ODS II column (4.5 mm by 25 cm) protected by a guard column (4 mm by 4.5 cm) packed with pellicular C_{18} reversed-phase material. The column effluent was monitored with a Perkin-Elmer LC-75 spectrophotometric detector (254 nm) and a Hewlett-Packard 3392A reporting integrator (Hewlett-Packard Co., Palo Alto, Calif.). The column was developed at a flow rate of 0.5 ml/min, with a 45-min, 9 to 30% linear gradient of methanol-chloroform (98/2, vol/vol), in 220 mM potassium phosphate, pH 4.0. Retention times for the standards were: CoASSG, 13.6 min; malonyl-CoA, 15.8 min; CoA, 17.8 min; succinyl-CoA, 27.8 min; acetoacetyl-CoA, 28.8 min; and acetyl-CoA, 29.5 min. ACP did not elute from the column under these conditions. The distribution of radioactivity in the chromatographic run was determined by collecting 0.1-ml (12 s) fractions and scintillation counting. Identity of the CoA thioesters was also confirmed by cochromatography of the ^{3}H-labeled CoA species with authentic ^{14}C-labeled thioesters. Recovery of CoA derivatives from the column was 75%.

RESULTS

Alteration of total CoA and ACP content. The specific cellular CoA content was altered by culturing small inocula of CoA-depleted strain SJ16 in media containing different concentrations of β-alanine as described in Materials and Methods. The concentrations were reproducibly varied between 58 and <1 pmol of CoA per 10^8 cells (Fig. 1A), which translates into total CoA concentrations of between 660 and <10 μM. The availability of CoA was the limiting factor for growth with the 0.0625, 0.125, and 0.25 μM β-alanine supplements, since strain SJ16 attained a density on these concentrations of only 0.5, 1.2 and 4.8×10^8 cells per ml, respectively. Higher β-alanine concentrations supported the growth of strain SJ16 to stationary phase (2.6×10^9 cells per ml).

Concomitant with the decrease in the cellular CoA concentration, the content of ACP also fell from a maximum of 15 to 2 pmol/10^8 cells. The decline in ACP content was not as dramatic as the drop in CoA (Fig. 1A), resulting in a different CoA/ACP ratio at each β-alanine concentration. The decrease in [*pan*-^{3}H]ACP (Fig. 1A) reflected a reduction in total ACP protein, since β-alanine-starved cells had lower ACP levels, as determined by a radioimmunoassay (data not shown) that used an antibody that cross-reacts equally well with both ACP and apo-ACP (12). Also, apo-ACP was not detected in strain SJ16 starved for β-alanine when leucine was used as the radiolabel (12).

Effects of CoA depletion. CoA depletion caused a change in the saturated/unsaturated fatty acid ratio in the membrane phospholipids (Fig. 1B). A progressive decrease in this ratio was observed at β-alanine concentrations below 2 μM (Fig. 1B). The change in this parameter was due to a decrease in the palmitic acid content with a corresponding increase in

the amount of *cis*-vaccinate. The mole percent of palmitoleic acid remained relatively unaffected.

The protein content of strain SJ16 was the same at all β-alanine concentrations tested (Fig. 1C). The phospholipid content was also constant except for the sharp increase observed at the three lowest β-alanine concentrations (Fig. 1C). These β-alanine supplements were growth limiting (see above), and thus it appeared that whereas protein synthesis and cell growth ceased at these low CoA levels, fatty acid and phospholipid production were not completely arrested. There was no significant difference in the phospholipid composition of strain SJ16 cultured on growth-limiting β-alanine as compared with that composition of the strain grown on a 4 μM β-alanine supplement (data not shown). The possibility that the accumulation of lipid was due to the uncoupling of protein and phospholipid biosynthesis in "relaxed" strains (23) was tested by determining the phospholipid/protein ratio in isogenic *rel*$^+$ and *relA* strains grown on the same range of β-alanine concentrations (Fig. 2). The phospholipid/protein ratio was not affected by the β-alanine supplement in strain SJ30 (*rel*$^+$), whereas the phospholipid content of strain SJ31 (*relA*) increased at the three lowest, growth-limiting concentrations of β-alanine (Fig. 2). Therefore, the increased phospholipid content of strains SJ16 and SJ31 after β-alanine starvation was attributed to the presence of the *relA* allele.

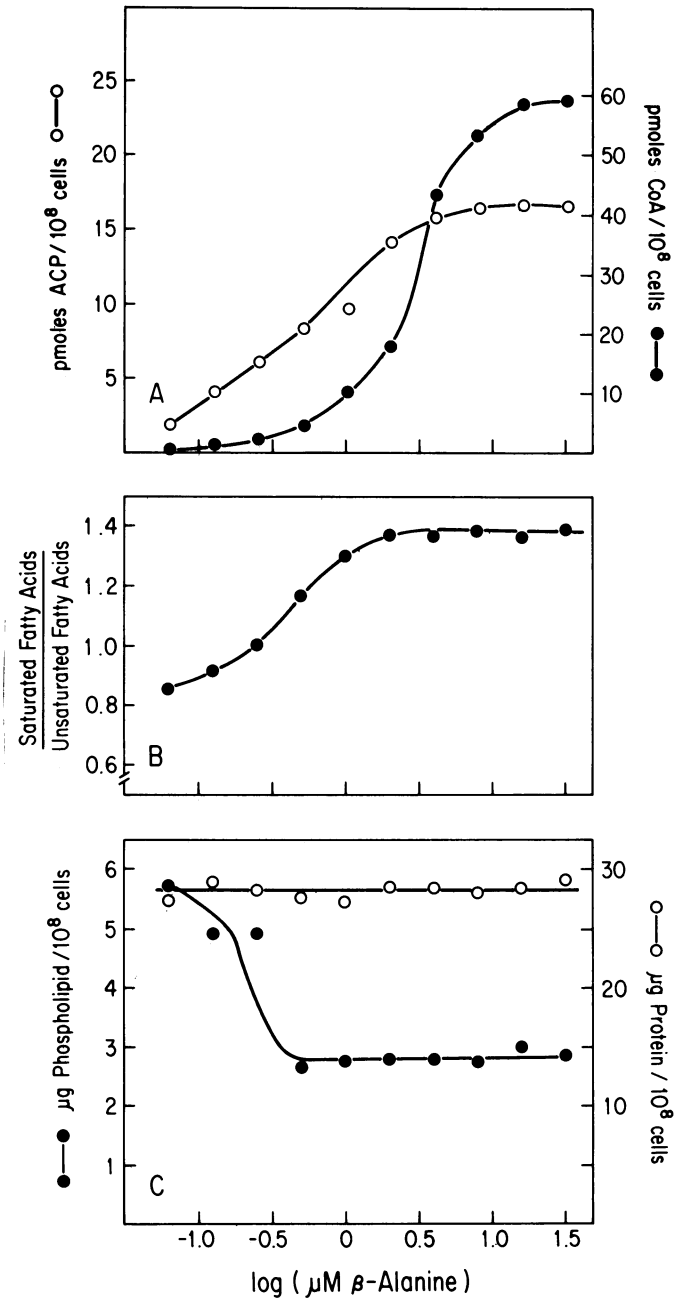

FIG. 1. Reduction of the intracellular CoA concentration and the production of phospholipid and protein by strain SJ16 (*panD2 relA1*). Strain SJ16 was grown in the presence of different β-alanine supplements to alter the specific cellular content of total CoA, as described in Materials and Methods. (A) Specific cellular content of CoA and ACP. (B) Saturated/unsaturated fatty acid ratio in membrane phospholipids. (C) Specific cellular content of phospholipid and protein.

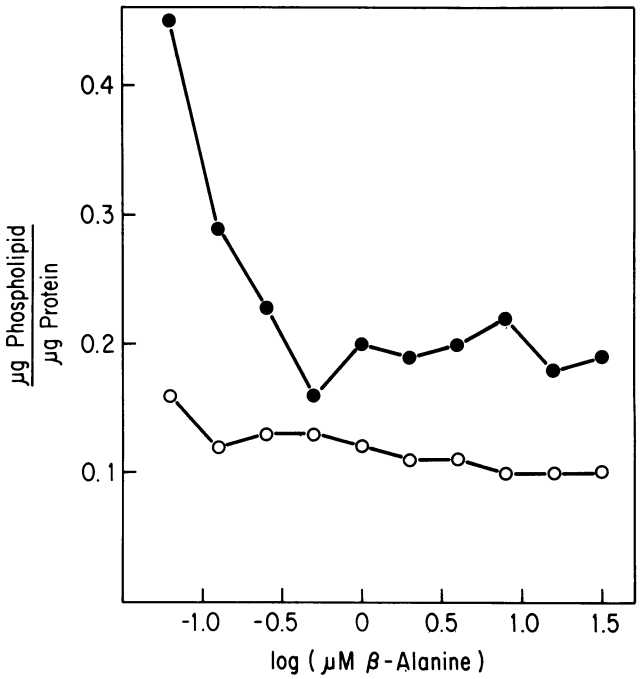

FIG. 2. Effect of CoA depletion on the phospholipid/protein ratio in strains SJ30 (*panD2 rel*$^+$) (○) and SJ31 (*panD2 relA1*) (●). Strains SJ30 and SJ31 were grown in the presence of different β-alanine supplements, as described in Materials and Methods.

The possibility that the alteration of the saturated/unsaturated fatty acid ratio was also due to the presence of the *relA* mutation was tested by determining the fatty acid composition of strains SJ30 and SJ31 starved for either β-alanine or amino acids (Table 1). Starvation for both β-alanine and amino acids resulted in an increase in the specific cellular phospholipid content in strain SJ31 (*relA*), but not in strain SJ30 (*rel*$^+$), whereas only β-alanine starvation affected the saturated/unsaturated fatty acid ratio (Table 1). These data show that the increase in the phospholipid content was due to the relaxed phenotype, and the decrease in the saturated/unsaturated fatty acid ratio was due to CoA depletion.

Composition of the CoA pool. Strain SJ16 was grown in the presence of 4 μM β-alanine on glucose minimal medium to a density of 4 × 10^8 cells per ml. Cells were harvested by

TABLE 1. Phospholipid content and fatty acid composition of strains SJ30 (*rel*$^+$) and SJ31 (*relA1*)

Strain	Starvation condition	Phospholipid (μg/10^8 cells)	saturated/ unsaturated fatty acid ratio
SJ30 (*rel*$^+$ *panD*)	None[a]	3.6	1.4
	β-Alanine[b]	3.9	0.9
	Met + Arg[c]	4.2	1.3
SJ31 (*relA1 panD*)	None	4.3	1.3
	β-Alanine	7.9	0.7
	Met + Arg	9.8	1.2

[a] Strains were grown to a density of 4×10^8 cells per ml in glucose minimal medium supplemented with 4 μM β-alanine, methione, and arginine.

[b] Strains were starved for β-alanine by growth in glucose minimal medium supplemented with only 0.0625 μM β-alanine, methionine, and arginine.

[c] At a density of 4×10^8 cells per ml, the bacteria were harvested, washed, and starved for methionine and arginine by resuspension in glucose minimal medium containing only 4 μM β-alanine. The medium was then incubated for 4 h at 37°C. The final density was 4.2×10^8 cells per ml.

centrifugation and washed with growth medium without β-alanine to remove the large excess of extracellular β-[3-^{3}H]alanine. It was important to include glucose in this washing step, because otherwise the amounts of CoA thioesters decreased significantly with a corresponding increase in unesterified CoA. On the other hand, the absence of β-alanine from the washing medium did not change the cellular CoA profile. The washed cell pellets were then extracted with 1 N formic acid in the cold. After neutralization, the distribution of radioactivity among the CoA species was analyzed by high-pressure liquid chromatography (Fig. 3). The CoA pool was composed primarily of four compounds: acetyl-CoA (79.8%), CoA (13.8%), succinyl-CoA (5.9%), and malonyl-CoA (0.5%) (Fig. 3A). There was no evidence for the presence of CoASSG.

The effect of CoA depletion on the composition of the CoA pool was studied by growing strain SJ16 on glucose minimal medium supplemented with 1 μM β-[3-^{3}H]alanine to a density of 3.4×10^8 cells per ml. The culture was filtered and washed with warm growth medium without β-alanine, and the cells were incubated in this medium for an additional 2 h at 37°C. The growth rate of the cells declined after 1 h of β-alanine starvation, and the CoA/ACP ratio dropped from about 1 before β-alanine removal to 0.35 after 2 h of starvation. The distribution of CoA species in cells grown on either 4 μM β-alanine (Fig. 3A) or 1 μM β-alanine (data not shown) was essentially the same before β-alanine starvation despite the reduced total CoA content (15 pmol/10^8 cells) of strain SJ16 grown in the presence of the lower β-alanine supplement. As in exponentially growing *E. coli* (Fig. 3A), acetyl-CoA was the predominant CoA species in β-alanine-starved bacteria (Fig. 3B), although the total CoA concentration dropped to 40 μM. Other than the precipitous drop in the total concentration of all CoA derivatives, the most notable change observed in CoA-depleted cells was the disappearance of succinyl-CoA (Fig. 3B).

DISCUSSION

Our results are consistent with the conclusion that the onset of growth stasis in CoA-depleted bacteria is due to their inability to synthesize amino acids and proteins. The uncoupling of protein and phospholipid synthesis in *relA* mutants allowed a direct test of this point. CoA depletion of a *relA* strain, but not its *rel*$^+$ counterpart, resulted in an increase in the phospholipid/protein ratio (Fig. 2), showing that a restricted supply of acetyl-CoA is more deleterious to protein synthesis than to fatty acid production. Applying a specific interpretation to this observation is difficult because of the complexity of intermediary metabolism. One possible explanation is that acetyl-CoA carboxylase may be better able to use low concentrations of acetyl-CoA than either citrate synthase or a number of other enzymes that draw on the acetyl-CoA pool for the synthesis of amino acids. Another possibility is that the onset of growth stasis and cessation of protein synthesis could be due to a restriction of energy production as a result of CoA depletion. However, this explanation seems unlikely, since phospholipid production continues in CoA-depleted cells (Fig. 1 and 2). Moreover, during aerobic growth on glucose, energy is generated primarily by glycolysis (which is CoA independent), whereas the tricarboxylic acid cycle (which is CoA dependent) operates to supply precursors for amino acid biosynthesis (28). The importance of tricarboxylic acid cycle intermediates as amino acid precursors is well established (10), and the disappearance of succinyl-CoA in β-alanine-starved cells (Fig. 3B) suggests that growth stasis ensues when the

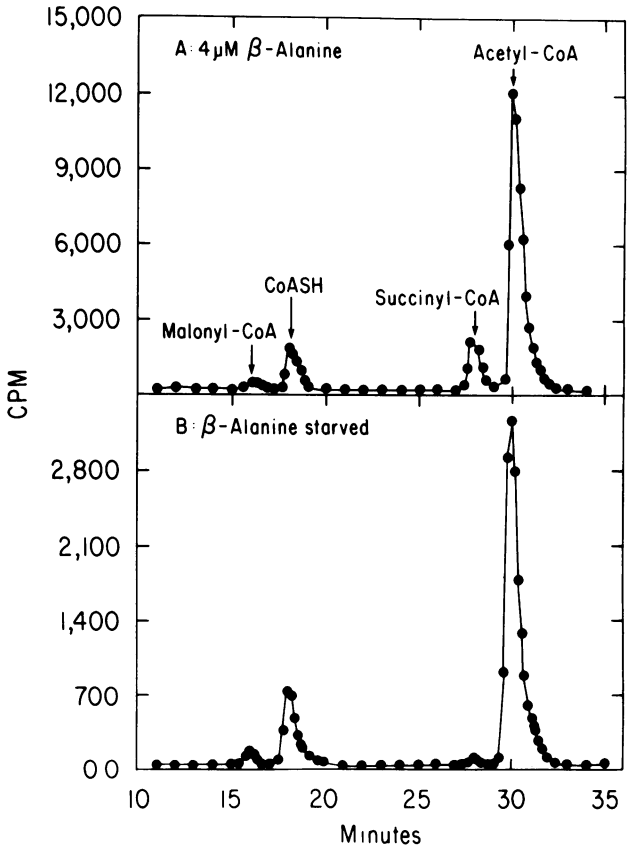

FIG. 3. Composition of the CoA pool. (A) CoA pool composition of strain SJ16 (*panD2*) sampled during exponential growth (4×10^8 cells per ml) on glucose minimal medium containing 4 μM β-[3-^{3}H]alanine. Total CoA content was 35.5 pmol/10^8 cells, and the CoA/ACP ratio was 4. (B) Total CoA content was reduced by growing strain SJ16 to a density of 3.4×10^8 cells per ml in glucose minimal medium supplemented with 1 μM β-[3-^{3}H]alanine. The cells were then harvested, washed, and resuspended in warm growth medium without β-alanine for 2 h. The total CoA content was 3.5 pmol/10^8 cells, and the CoA/ACP ratio was 0.35.

acetyl-CoA concentration falls below the level required to maintain the operation of this cycle. One explanation for this finding is that all of the α-ketoglutarate derived from the condensation of acetyl-CoA and oxaloacetate is being funneled into protein via glutamate rather than being converted to succinyl-CoA. Alternatively, CoA depletion may prevent the operation of the tricarboxylic acid cycle by altering the intracellular concentration of key allosteric modifiers.

Four CoA species composed the bulk of the CoA pool in *E. coli*. Acetyl-CoA was the major thioester, reaching a concentration of 0.3 mM in strain SJ16 as it grew exponentially in glucose minimal medium plus 4 μM β-alanine. Acetyl-CoA is also the major CoA derivative in *Salmonella typhimurium* LT2 grown in glucose minimal medium, at a concentration estimated at 0.23 mM (3). Nonesterified CoA and succinyl-CoA were also abundant, whereas malonyl-CoA was a minor species. The lack of CoASSG in our samples directly contradicts the numerous data of Loewen (16–19), who reported that the concentration of CoASSG ranges between 42 and 95% of the total CoA pool, depending on the physiological state of the cell. This discrepancy can be attributed to improper sample handling, since CoASSG was quantitated in those studies after ion-exchange chromatography at alkaline pH (ca. pH 8.5), thus promoting the artifactual formation of mixed disulfides. In our experience, formic acid extracts are stable for 1 week when stored at −70°C, but after the pH is elevated for liquid chromatography, an increase in CoASSG is observed when the same sample is analyzed on subsequent days. Consistent with this conclusion, King and Reiss recently reported a 300% increase in CoASSG 12 h after the pH of the acid extracts was adjusted to pH 6.0 (15).

ACP biogenesis appears to be controlled in part by the supply of precursors to its 4′-phosphopantetheine prosthetic group. Since apo-ACP does not accumulate in β-alanine-starved cells (12), and both ACP and apo-ACP are metabolically stable (12, 21), the decrease in ACP content observed in CoA-depleted cells (Fig. 1A) suggests that CoA may be required at some stage of ACP gene expression. This decrease in ACP content may explain the changes in fatty acid metabolism observed in *panD* strains grown in the presence of low β-alanine supplements. The condensing enzymes are known to play a key role in regulating the saturated/unsaturated fatty acid ratio in membrane phospholipids (7), and the compositional change that occurs in CoA-depleted cells suggests that the activity of these enzymes is affected. 3-Ketoacyl-ACP synthase I catalyzes the committed step in unsaturated fatty acid biosynthesis (7), and one possibility may be that this enzyme has a higher affinity for ACP thioesters than competing enzymes, thus favoring the synthesis of unsaturated fatty acids at low intracellular ACP concentrations. Further experiments will be required to clearly establish the relationships between CoA, ACP, and fatty acid biosynthesis.

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