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# Involvement of long-chain acyl coenzyme A for lipid synthesis in repression of acetyl-coenzyme A carboxylase in *Candida lipolytica*

(mutants/acyl-CoA synthetase I/acyl-CoA synthetase II/two long-chain acyl-CoA pools/regulation of fatty acid synthesis)

TATSUYUKI KAMIRYO, YOSHIKI NISHIKAWA, MASAYOSHI MISHINA, MINEKO TERAO, AND SHOSAKU NUMA

Department of Medical Chemistry, Kyoto University Faculty of Medicine, Kyoto 606, Japan

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ABSTRACT **Mutant strains of *Candida lipolytica* defective in acyl-CoA synthetase II [acid:CoA ligase (AMP-forming), EC 6.2.1.3] have been isolated. The mutants fail to grow on fatty acid as a sole carbon source but are capable of incorporating exogenous fatty acid into cellular lipids. This observation, together with our previous finding that mutant strains defective in acyl-CoA synthetase I cannot incorporate exogenous fatty acid into cellular lipids but are able to degrade fatty acid via β-oxidation, indicates the presence of two functionally distinct long-chain acyl-CoA pools in the cell—i.e., one for lipid synthesis and the other for β-oxidation. Unlike the wild-type and the revertant strains as well as the mutants lacking acyl-CoA synthetase II, the mutants defective in acyl-CoA synthetase I do not exhibit the repression of acetyl-CoA carboxylase [acetyl-CoA: carbon-dioxide ligase (ADP-forming), EC 6.4.1.2] by exogenous fatty acid. Measurement of the two long-chain acyl-CoA pools with the aid of appropriate mutant strains has indicated that the long-chain acyl-CoA to be utilized for lipid synthesis, but not that to be degraded via β-oxidation, is involved in the repression of acetyl-CoA carboxylase.**

Acetyl-CoA carboxylase [acetyl-CoA:carbon-dioxide ligase (ADP-forming), EC 6.4.1.2], which mediates the initial step in the biosynthesis of long-chain fatty acids, plays a critical role in the regulation of this synthetic process (1). For the purpose of studying the mechanism responsible for the regulation of the cellular content of this enzyme, *Candida lipolytica* represents a suitable eukaryotic system because this yeast is capable of utilizing fatty acid (or *n*-alkane) as well as glucose as a sole carbon source. Studies with specific antibody to *C. lipolytica* acetyl-CoA carboxylase have demonstrated that the content of this enzyme is markedly decreased in cells grown on fatty acid compared with cells grown on glucose (2). It has also been shown by combined immunochemical and isotopic techniques that the decrease in acetyl-CoA carboxylase content in fatty acid-grown cells is due to diminished synthesis of the enzyme (2).

Our previous work has demonstrated that mutant strains of *Saccharomyces cerevisiae* defective in long-chain acyl-CoA synthetase [acid:CoA ligase (AMP-forming), EC 6.2.1.3], in contrast to the wild-type strain, exhibit little repression of acetyl-CoA carboxylase when cells are grown in medium supplemented with fatty acid, thus indicating that the activation of exogenous fatty acid is required for the repression of acetyl-CoA carboxylase (3). In an attempt to gain further insight into the mechanism underlying the repression of acetyl-CoA carboxylase, we isolated mutant strains of *C. lipolytica* that apparently exhibited no long-chain acyl-CoA synthetase activity (4). Because the activation of exogenous fatty acid is an obligatory step for its further metabolism, it was unexpected that the mutant strains isolated were capable of growing on fatty acid (or *n*-alkane) as a sole carbon source. This phenotype has been understood, however, by the finding that the mutant strains, unlike the wild-type strain, cannot incorporate exogenous fatty acid as a whole into cellular lipids but are able to degrade it to yield acetyl-CoA, from which cellular fatty acids are synthesized *de novo* (4). Moreover, this finding has led to the discovery of a second long-chain acyl-CoA synthetase which occurs in the mutant strains as well as in the wild-type strain (5). This enzyme, designated as acyl-CoA synthetase II, requires phosphatidylcholine for its activity and can be separated from phosphatidylcholine-independent acyl-CoA synthetase I which is absent in the mutant strains (5).

Thus, it has been concluded that acyl-CoA synthetase I is responsible for the production of long-chain acyl-CoA to be utilized for the synthesis of cellular lipids, whereas acyl-CoA synthetase II provides long-chain acyl-CoA that is exclusively degraded via β-oxidation (4, 5). Consistent with this conclusion is the fact that, in contrast to acyl-CoA synthetase I, acyl-CoA synthetase II is induced by fatty acid and exhibits a broad substrate specificity with respect to fatty acid (5). In further support of the different physiological roles of the two long-chain acyl-CoA synthetases, their subcellular localizations are different (6). Acyl-CoA synthetase I is distributed among various subcellular fractions, including microsomes and mitochondria where glycerophosphate acyltransferase is located, whereas acyl-CoA synthetase II is localized in microbodies where the acyl-CoA-oxidizing system is located.

From these findings, it may be inferred that the cell of *C. lipolytica* has at least two long-chain acyl-CoA pools, one destined for lipid synthesis and the other for β-oxidation. The present investigation was designed to prove the presence of the two distinct long-chain acyl-CoA pools and to determine which of these pools is involved in the repression of acetyl-CoA carboxylase. For this purpose, we isolated additional mutant strains of *C. lipolytica*, including mutants defective in acyl-CoA synthetase II. Studies with these mutants as well as with the acyl-CoA synthetase I mutants previously isolated have indicated that two functionally independent long-chain acyl-CoA pools in fact exist and that acetyl-CoA carboxylase is repressed when the long-chain acyl-CoA to be utilized for lipid synthesis accumulates in the cell.

## MATERIALS AND METHODS

**Yeast Strains.** *C. lipolytica* NRRL Y-6795, a haploid yeast, was used as a wild-type strain. Mutant strains L-5 and L-7, which were defective in acyl-CoA synthetase I, were derived from the wild-type strain as described (4). Strains RL7-1 and RL7-2 were spontaneous revertants obtained from L-7 (4). Other mutant strains were isolated as described below.

**Reagents, Media, and Culture.** Phosphate acetyltransferase [acetyl-CoA:orthophosphate acetyltransferase, EC 2.3.1.8] from *Clostridium kluyveri* and citrate(*si*)-synthase [citrate oxaloa-

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cetate-lyase (*pro*-3S-$CH_2COO^-$ → acetyl-CoA), EC 4.1.3.7] and malate dehydrogenase [L-malate:$NAD^+$ oxidoreductase, EC 1.1.1.37] from pig heart were products of Boehringer Mannheim. [*U*-$^{14}$C]Palmitic acid and $NaH^{14}CO_3$ were purchased from the Radiochemical Centre (Amersham, England). Other reagents were obtained as described (4, 5). N medium contained 0.67% Bacto-yeast nitrogen base (Difco), 0.5% $KH_2PO_4$, and 0.5% $K_2HPO_4$. Acidic NB medium was composed of 0.67% Bacto-yeast nitrogen base, 1% $KH_2PO_4$, and 1% Brij 58 (Kao-Atlas, Tokyo). NB medium, B medium, and YPG medium were as described (4). Agar plates contained 2% Bacto-agar (Difco) in addition to the components of the medium indicated. Cells were grown aerobically at 25°C. The cellular growth in liquid media was monitored turbidimetrically as described (2).

**Isolation of Mutants.** Mutant strains LA-445, LA-633, and LB-742 were isolated from L-7 by selecting strains incapable of degrading fatty acid as follows. L-7 cells were mutagenized with 3% ethyl methanesulfonate as described (7) and were treated at 25°C for 1 hr with nystatin (50 μg/ml; Sigma) in acidic NB medium containing 0.2% oleic acid according to Snow (8) in order to enrich mutants with the desired phenotype. The surviving cells were plated on YPG agar and then replica-plated onto NB agar containing 0.2% oleic acid and onto N agar supplemented with 0.4% sodium acetate. Strains that failed to grow on oleic acid but did grow on acetic acid were selected. Subsequently, two groups of spontaneous revertant strains were isolated as follows. Revertants of the first group were selected as strains capable of growing in the presence of cerulenin (a generous gift from S. Ōmura and J. Awaya, Kitasato University, Tokyo) and fatty acid as described (4). Among these were strains A-445 and A-633-7 (derived from LA-445 and LA-633, respectively) which grew normally in B medium containing 2% glucose and 1% oleic acid. Revertants of the second group, L-445 and L-633 (derived from LA-445 and LA-633, respectively), were isolated by selecting strains capable of growing on NB agar containing 0.2% oleic acid.

**Assay of Enzymes.** Acyl-CoA synthetase activity was assayed either by the hydroxamic acid method or by the isotopic method as described (5). For screening mutant strains defective in acyl-CoA synthetase II, cells grown overnight in YPG medium containing 1% oleic acid were treated with toluene essentially according to Serrano *et al.* (9) and tested for acyl-CoA synthetase activity by the hydroxamic acid method in the presence of 4 mM oleic acid and 0.04% Triton X-100. The activities of acyl-CoA synthetase I and acyl-CoA synthetase II in the particulate fraction (230,000 × *g* pellet) were determined by measuring the phosphatidylcholine-independent and the phosphatidylcholine-dependent activities, respectively, by the isotopic method. Acyl-CoA-oxidizing activity in the crude extract (for preparation, see ref. 5) was determined spectrophotometrically by measuring the palmityl-CoA-dependent reduction of $NAD^+$ as described (6). Acetyl-CoA carboxylase activity in the gel-filtered soluble fraction was assayed by the $H^{14}CO_3^-$ fixation method as described (2); values for the acid-stable radioactivity were corrected for the quenching caused by the polyethylene glycol derived from the reaction mixture.

**Immunochemical Procedures.** Rabbit antiserum was prepared against homogeneous *C. lipolytica* acyl-CoA synthetase I (10) as well as against homogeneous *C. lipolytica* acetyl-CoA carboxylase (11), and the immunoglobulin fraction was isolated as described (12). The procedure of immunochemical titrations was as described (2, 10).

**Determination of Long-Chain Acyl-CoA.** Long-chain acyl-CoA contents of yeast cells were determined essentially according to the procedure of A. Olbrich and F. Lynen (personal communication). To approximately 1 g (wet weight) of yeast cells, 5 ml of 6% (wt/vol) perchloric acid containing 2 mM EDTA was added. The following operations were carried out at 0–4°C unless otherwise specified. The cell suspension was sonicated with a Branson W185D sonifier equipped with a microtip at maximal output for 2 min, and the homogenate was centrifuged at 34,800 × *g* for 10 min. The resulting precipitate was washed twice with 5 ml of 6% perchloric acid containing 2 mM EDTA as described above, except that the sonication was performed for 1 min at the first washing and was omitted at the second washing. The washed precipitate was suspended in 1 ml of 25 mM dithiothreitol. The suspension was adjusted to pH 12 by the addition of 1 M KOH and incubated at 30°C for 30 min to hydrolyze the thioester bond of long-chain acyl-CoA. The mixture was then adjusted to pH 5 by the addition of 6% perchloric acid and centrifuged at 34,800 × *g* for 10 min. The resulting supernatant was assayed for CoA by the catalytic method of Michal and Bergmeyer (13) with the aid of phosphate acetyltransferase, citrate (*si*)-synthase, and malate dehydrogenase.

**Analytical Methods.** Fatty acids from cells grown in B medium containing 2% glucose, 0.5% pentadecanoic acid, and 1% Brij 58 were analyzed by gas/liquid chromatography as described (4, 14). Protein was determined by the method of Lowry *et al.* (15) with bovine serum albumin (Sigma) as a standard.

## RESULTS

**Presence of Two Long-Chain Acyl-CoA Pools.** To examine whether functionally distinct long-chain acyl-CoA pools exist, we attempted to isolate mutant strains of *C. lipolytica* defective in acyl-CoA synthetase II. Because acyl-CoA synthetase II is responsible for the production of long-chain acyl-CoA that is degraded via β-oxidation (4, 5) and because previous work has not excluded the possibility that a portion of the long-chain acyl-CoA produced by acyl-CoA synthetase I can also serve as substrate of the β-oxidation system, we decided to isolate acyl-CoA synthetase II mutants as double-mutant strains unable to degrade fatty acid, starting with strain L-7 which is an acyl-CoA synthetase I mutant isolated previously (4). Strains that failed to grow on oleic acid but did grow on acetic acid were isolated and tested for acyl-CoA synthetase activity in toluenized cells in the presence of phosphatidylcholine. LA-445 and LA-633 were among the strains that showed little acyl-CoA synthetase activity, thus apparently representing mutants defective in both acyl-CoA synthetase I and acyl-CoA synthetase II.

An attempt was next made to isolate mutant strains that were defective in acyl-CoA synthetase II but had intact acyl-CoA synthetase I by selecting revertant strains capable of incorporating exogenous fatty acid into cellular lipids—i.e., strains able to grow in the presence of cerulenin [an inhibitor of fatty acid synthetase (16)] and fatty acid. Such revertant strains, A-445 and A-633-7, were spontaneously isolated from LA-445 and LA-633, respectively. The ability of A-445 and A-633-7 to incorporate exogenous fatty acid into cellular lipids was confirmed by the fact that cells of these strains, in contrast to cells of LA-445 and LA-633 as well as of L-445 and L-633 (see below), contained predominantly odd-chain-length fatty acids when they were grown on glucose plus pentadecanoic acid (see ref. 4).

On the other hand, strains L-445 and L-633, derived from LA-445 and LA-633, respectively, were spontaneous revertants capable of growing on oleic acid, thus apparently representing revertants that had intact acyl-CoA synthetase II but were defective in acyl-CoA synthetase I. LB-742 belonged to another

group of double-mutant strains that were unable to grow on oleic acid but exhibited normal acyl-CoA synthetase activity in toluenized cells.

LA-445 and LA-633 exhibited neither acyl-CoA synthetase I nor acyl-CoA synthetase II activity; A-445 and A-633-7 were defective only in acyl-CoA synthetase II activity; and L-445 and L-633 were defective only in acyl-CoA synthetase I activity (Table 1). LB-742 lacked acyl-CoA-oxidizing activity and acyl-CoA synthetase I activity. The acyl-CoA synthetase activity of A-445 and A-633-7 cells was almost completely inhibited by incubation with antibody specific to acyl-CoA synthetase I, whereas that of L-445 and L-633 cells as well as of L-7 and LB-742 cells was unaffected. This verified that the enzyme absent from A-445 and A-633-7 is acyl-CoA synthetase II.

Strains A-445 and A-633-7, which were defective in acyl-CoA synthetase II but had intact acyl-CoA synthetase I, completely failed to grow on fatty acid as a sole carbon source as did the double-mutant strains LA-445 and LA-633, which lacked both acyl-CoA synthetases. In contrast, the revertant strains L-445 and L-633, which had regained acyl-CoA synthetase II but were defective in acyl-CoA synthetase I, grew normally on fatty acid. These results indicate that the long-chain acyl-CoA to be degraded via $\beta$-oxidation is provided by acyl-CoA synthetase II but not by acyl-CoA synthetase I. This finding, together with the inability of the acyl-CoA synthetase I mutants to incorporate exogenous fatty acid as a whole into cellular lipids (4), leads to the conclusion that the long-chain acyl-CoA produced by acyl-CoA synthetase I is utilized solely for lipid synthesis and that produced by acyl-CoA synthetase II is destined exclusively for $\beta$-oxidation. Thus, the presence of two distinct long-chain acyl-CoA pools which do not take the place of each other is clearly indicated.

**Acyl-CoA Synthetase Required for the Repression of Acetyl-CoA Carboxylase.** In order to determine which of the acyl-CoA synthetases is involved in the repression of acetyl-CoA carboxylase by exogenous fatty acid, we determined the levels of acetyl-CoA carboxylase activity in cells of the different mutant and revertant strains as well as of the wild-type strain that were grown on glucose, oleic acid, or glucose plus oleic acid. All the strains examined showed similar activities of acetyl-CoA carboxylase when they were grown on glucose (Table 2). The activity of acetyl-CoA carboxylase in the wild-type strain (Y-6795) as well as in the mutant strains defective in acyl-CoA synthetase II (A-445 and A-633-7) and in the revertant strains (RL7-1 and RL7-2) derived from an acyl-CoA synthetase I mutant was markedly lowered by the addition of oleic acid to the culture medium regardless of the simultaneous presence of glucose. In contrast, the activity of acetyl-CoA carboxylase in the mutant strains defective in acyl-CoA synthetase I (L-5 and L-7) was hardly changed when they were grown in oleic acid-containing media.

Table 1. Activities of acyl-CoA synthetase I, acyl-CoA synthetase II, and acyl-CoA oxidation in cells grown on glucose plus oleic acid

| Strain | Activity, nmol/min per mg of protein: Acyl-CoA synthetase I | Acyl-CoA synthetase II | Acyl-CoA oxidation* |
|---|---|---|---|
| Y-6795 | 30.4 | 27.5 | 67 |
| L-7 | 2.3 | 26.7 | 39 |
| LA-445 | 3.1 | 0.2 | 42 |
| LA-633 | 1.8 | 0.0 | 48 |
| A-445 | 32.6 | 0.0 | 50 |
| A-633-7 | 35.6 | 0.0 | 41 |
| L-445 | 2.0 | 30.3 | 44 |
| L-633 | 1.1 | 29.4 | 62 |
| LB-742 | 2.7 | 38.0 | 0 |

Cells were grown in B medium containing 2% glucose plus 1% oleic acid and were harvested at the midlogarithmic phase.
* Expressed as NADH formed.

Table 2. Acetyl-CoA carboxylase activity in wild-type, mutant, and revertant cells grown on different carbon sources

| Strain | Activity (nmol/min per mg of protein) in cells grown on: Glucose | Oleic acid | Glucose plus oleic acid |
|---|---|---|---|
| Y-6795 | 40.6 | 4.9 | 5.4 |
| A-445 | 36.0 | — | 2.0 |
| A-633-7 | 43.7 | — | 6.1 |
| L-5 | 35.7 | 30.3 | — |
| L-7 | 39.6 | 28.1 | 33.2 |
| RL7-1 | 43.9 | 4.9 | — |
| RL7-2 | 43.5 | 4.8 | 4.6 |

Cells were grown in B medium containing 2% glucose or 1% oleic acid or 2% glucose plus 1% oleic acid and were harvested at the midlogarithmic phase.

Immunochemical titrations demonstrated that the amount of acetyl-CoA carboxylase activity inhibited by a fixed amount of the specific antibody was identical for all the enzyme preparations examined (Fig. 1), indicating that the observed variations in the activity level of the enzyme were in fact due to corresponding changes in the amount of the enzyme protein.

Thus, it is evident that the activity of acyl-CoA synthetase I, but not that of acyl-CoA synthetase II, is required for the repression of acetyl-CoA carboxylase. Moreover, the failure of the acyl-CoA synthetase I mutants to repress acetyl-CoA carboxylase, together with the normal response of the revertant strains, indicates that a single mutation causes both the lack of acyl-CoA synthetase I and the derepression of acetyl-CoA carboxylase.

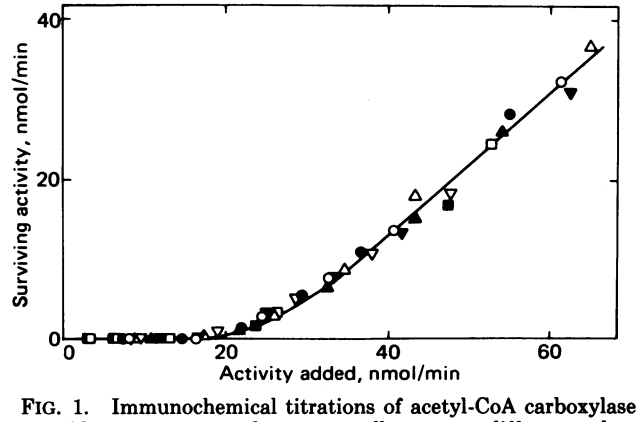


FIG. 1. Immunochemical titrations of acetyl-CoA carboxylase from wild-type, mutant, and revertant cells grown on different carbon sources. Cells were grown in B medium containing 2% glucose or 1% oleic acid or 2% glucose plus 1% oleic acid and were harvested at the midlogarithmic phase. Increasing amounts of the gel-filtered soluble fraction containing the indicated activities of acetyl-CoA carboxylase were added to 53 $\mu$g of anti-acetyl-CoA carboxylase immunoglobulin. After incubation at 0°C for 3 hr, the surviving enzyme activity was determined. The specific activities (nmol/min per mg of protein) of the enzyme preparations used were as follows: Y-6795 cells grown on glucose (○), 43.7; Y-6795 cells grown on oleic acid (●), 4.3; A-633-7 cells grown on glucose (□), 51.3; A-633-7 cells grown on glucose plus oleic acid (■), 10.3; L-7 cells grown on glucose (△), 42.0; L-7 cells grown on oleic acid (▲), 32.0; RL7-2 cells grown on glucose (▽), 44.7; RL7-2 cells grown on oleic acid (▼), 5.1.

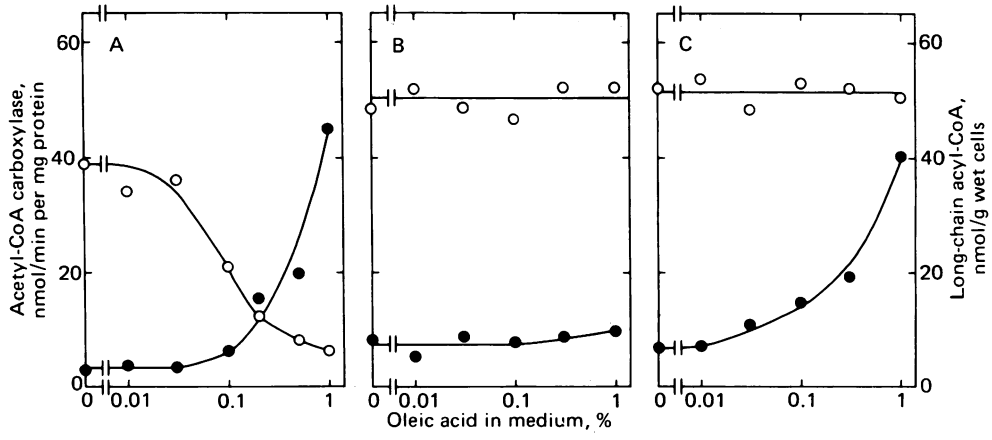


FIG. 2. Levels of long-chain acyl-CoA and acetyl-CoA carboxylase in cells grown in the presence of increasing concentrations of oleic acid. Cells of A-633-7 (*A*), LA-633 (*B*), and LB-742 (*C*) were grown in B medium containing oleic acid at the indicated concentrations and 2% glucose. The cells were harvested at midlogarithmic phase and were divided into two portions, for the determinations of long-chain acyl-CoA content (●) and acetyl-CoA carboxylase activity (○).

**Long-Chain Acyl-CoA Pool Involved in the Repression of Acetyl-CoA Carboxylase.** The results described above suggest that the long-chain acyl-CoA formed by acyl-CoA synthetase I may be responsible for the repression of acetyl-CoA carboxylase. Therefore, with the aid of appropriate mutant strains, an attempt was made to measure separately the two distinct long-chain acyl-CoA pools provided by acyl-CoA synthetase I and acyl-CoA synthetase II. When grown on glucose plus fatty acid, cells of a mutant defective in acyl-CoA synthetase II (e.g., A-633-7) should accumulate the long-chain acyl-CoA to be utilized for lipid synthesis whereas cells of a mutant lacking both acyl-CoA synthetase I and the acyl-CoA-oxidizing system (e.g., LB-742) should accumulate the long-chain acyl-CoA destined for β-oxidation. On the other hand, a mutant defective in both acyl-CoA synthetases (e.g., LA-633) should produce no long-chain acyl-CoA from exogenous fatty acid.

In the experiment represented in Fig. 2, strain A-633-7 and strain LB-742, in contrast to strain LA-633, exhibited a remarkable accumulation of long-chain acyl-CoA when they were grown in the presence of higher concentrations of oleic acid; LB-742, instead of L-7 (defective only in acyl-CoA synthetase I), was used because no significant accumulation of long-chain acyl-CoA (5.3 nmol/g of wet cells) was observed in L-7 cells grown in the presence of 1% oleic acid. On the other hand, the repression of acetyl-CoA carboxylase was observed in A-633-7 cells, but not in LB-742 and LA-633 cells, when they were grown in the presence of higher concentrations of oleic acid. These results indicate that the long-chain acyl-CoA produced by acyl-CoA synthetase I (i.e., that to be utilized for lipid synthesis) is causally related to the repression of acetyl-CoA carboxylase, whereas the long-chain acyl-CoA produced by acyl-CoA synthetase II (i.e., that to be degraded via β-oxidation) is not involved in this repression. Furthermore, this finding provides additional evidence for the presence of two independent long-chain acyl-CoA pools.

## DISCUSSION

The present investigation provides several lines of evidence indicating that there are two functionally distinct long-chain acyl-CoA pools in the cell of *C. lipolytica*—i.e., one for lipid synthesis and the other for β-oxidation. First, the long-chain acyl-CoA produced by acyl-CoA synthetase I does not undergo β-oxidation but is utilized for lipid synthesis. Second, the repression of acetyl-CoA carboxylase involves the long-chain acyl-CoA formed by acyl-CoA synthetase I but not that formed by acyl-CoA synthetase II. Third, the long-chain acyl-CoA generated by acyl-CoA synthetase I accumulates when cells are grown in the presence of exogenous fatty acid, whereas the accumulation of the long-chain acyl-CoA produced by acyl-CoA synthetase II does not occur unless β-oxidation is blocked. In addition, our previous work has demonstrated that the long-chain acyl-CoA produced by acyl-CoA synthetase II is not utilized for lipid synthesis but is degraded via β-oxidation (4) and that acyl-CoA synthetase I and acyl-CoA synthetase II are localized at the subcellular sites where lipid synthesis and β-oxidation take place, respectively (6). From all these findings, it is concluded that the long-chain acyl-CoA provided by acyl-CoA synthetase I is utilized solely for lipid synthesis and that provided by acyl-CoA synthetase II is destined exclusively for β-oxidation.

The finding that the long-chain acyl-CoA formed by acyl-CoA synthetase I, but not that formed by acyl-CoA synthetase II, is involved in the repression of acetyl-CoA carboxylase is evidently of teleological significance in view of the homeostasis of lipid synthesis. The long-chain acyl-CoA destined for lipid synthesis is supplied both by fatty acid synthesis *de novo*, the rate of which is regulated by acetyl-CoA carboxylase, and by the activation of exogenous fatty acid catalyzed by acyl-CoA synthetase I. The results of the present investigation do not exclude the possibility that the immediate mediator of the repressive effect on acetyl-CoA carboxylase is a compound that is metabolically derived from the long-chain acyl-CoA to be utilized for lipid synthesis.

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