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Biochemistry

Candida lipolytica mutants defective in an acyl-coenzyme A synthetase: Isolation and fatty acid metabolism

(cerulenin/composition of cellular fatty acids/Schmidt reaction/single-cell protein)

TATSUYUKI KAMIRYO, MASAYOSHI MISHINA, SHIN-ICHI TASHIRO, 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 an acyl-CoA synthetase [acid:CoA ligase (AMP-forming); EC 6.2.1.3] were isolated. The mutant strains apparently exhibited no acyl-CoA synthetase activity *in vitro* and were, in contrast to the wild-type strain, incapable of growing in the presence of exogenous fatty acid when cellular synthesis *de novo* of fatty acid was blocked. However, the mutant strains grew on either fatty acid or *n*-alkane as a sole carbon source at rates comparable to that observed for the wild-type strain. Analysis of the fatty acid composition of the lipids from the mutant cells grown on odd-chain-length fatty acid as well as [^{14}C]oleic acid incorporation studies have shown that the mutant cells, unlike the wild-type cells, cannot incorporate exogenous fatty acid as a whole into cellular lipids, but utilize the fatty acid that is synthesized *de novo* from acetyl-CoA produced by degradation of exogenous fatty acid. This finding indicates the presence of at least two acyl-CoA synthetases that activate long-chain fatty acid. One, designated acyl-CoA synthetase I, which is absent in the mutant strains, is responsible for the production of acyl-CoA to be utilized for the synthesis of cellular lipids. The other acyl-CoA synthetase provides acyl-CoA that is exclusively degraded via β-oxidation to yield acetyl-CoA.**

In our previous work, mutant strains of *Saccharomyces cerevisiae* defective in acyl-CoA synthetase [acid:CoA ligase (AMP-forming); EC 6.2.1.3] were isolated and characterized (1, 2). Studies with these mutants have indicated that acyl-CoA synthetase activity is required for the repression of acetyl-CoA carboxylase [acetyl-CoA:carbon-dioxide ligase (ADP-forming); EC 6.4.1.2] by exogenous fatty acid (1). In the meantime, it has become evident that *Candida lipolytica* provides a more suitable system for studying the repression of acetyl-CoA carboxylase than *S. cerevisiae*. We have demonstrated that the acetyl-CoA carboxylase content of *C. lipolytica* cells grown on fatty acid or *n*-alkane as a sole carbon source is remarkably lowered as compared with that of glucose-grown cells and that this decrease in the enzyme content is due to diminished synthesis of the enzyme (3). In hopes of gaining further insight into the molecular mechanism underlying the repression of acetyl-CoA carboxylase, we attempted to isolate acyl-CoA synthetase mutants of *C. lipolytica*. Unexpectedly, several such mutants isolated were capable of growing on fatty acid or *n*-alkane as a sole carbon source despite the apparent lack of acyl-CoA synthetase activity. This has prompted us to study the mechanism by which fatty acid is metabolized in these mutant cells. In the present investigation, we have demonstrated that the mutant strains, unlike the wild-type strain, cannot incorporate exogenous fatty acid as a whole into cellular lipids, but utilize the fatty acid that is synthesized *de novo* from acetyl-CoA produced by degradation of exogenous fatty acid. This finding indicates that the wild-type strain of *C. lipolytica* possesses at least two distinct long-chain acyl-CoA synthetases, one of which is absent in the mutant strains.

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MATERIALS AND METHODS

Yeast Strains. A haploid yeast, *Candida lipolytica* NRRL Y-6795, was used as a wild-type strain. Mutant strains L-5 and L-7, as well as revertant strains RL7-1, RL7-2, and RL7-8, were isolated as described below.

Chemicals, Media, and Culture. CoA and ATP were obtained from Boehringer (Mannheim, Germany). Fatty acids and *n*-alkanes were purchased from Nakarai (Kyoto, Japan). NB medium was composed (wt/wt) of 0.67% Bacto-yeast nitrogen base (Difco, Detroit, MI), 0.5% KH_2PO_4, 0.5% K_2HPO_4, and 1% Brij 58 (Kao-Atlas, Tokyo, Japan). B medium contained (wt/wt) 0.1% Bacto-yeast extract (Difco), 0.5% $NH_4H_2PO_4$, 0.25% KH_2PO_4, 0.01% NaOH, 0.1% $MgSO_4 \cdot 7H_2O$, and 0.0001% $FeCl_3 \cdot 6H_2O$. YPG medium was composed (wt/wt) of 0.3% Bacto-yeast extract, 0.5% Bacto-peptone (Difco), 2% glucose, 0.5% KH_2PO_4, and 0.5% K_2HPO_4. Agar plates contained 2% Bacto-agar (Difco) in addition to the components of the medium indicated. SB solution consisted of 0.1 M NaCl, 10 mM $MgCl_2$, and 1% Brij 58. Cells were grown aerobically at 25°. The cellular growth in liquid media was monitored by an Akiyama D. S. spectrometer (Fuji, Tokyo, Japan) at either 570 or 660 nm.

Isolation of Mutants. NRRL Y-6795 cells grown in YPG medium were mutagenized with 3% ethyl methanesulfonate (Nakarai) according to the method of Lindegren *et al.* (4). The survival rate was 40–60%. The mutagenized cells were treated with 50 μg of nystatin per ml (Sigma, St. Louis, MO) in the presence of 0.2% oleic acid for 1 hr according to the procedure of Snow (5). The surviving cells were grown in NB medium containing 2% glucose for 40 hr, plated on NB agar plates containing 2% glucose, and replica-plated onto YPG agar and YPG agar containing 68 μM cerulenin (a generous gift from S. Ōmura and J. Awaya, Kitasato University, Tokyo, Japan), 0.01% palmitic acid, 0.01% oleic acid, and 1% Brij 58. Strains that failed to grow on the latter agar plate were tested for acyl-CoA synthetase activity in toluenized cells as described (1). Spontaneous revertants were selected either in YPG medium containing 0.68 mM cerulenin, 0.025% palmitic acid, 0.025% oleic acid, and 1% Brij 58 or on YPG agar containing the same ingredients; the concentration of cerulenin was raised to exclude phenotypic revertants that were resistant to cerulenin. In subsequent experiments analogous mutants were obtained without nystatin treatment.

Assay of Acyl-CoA Synthetase. Cells were disrupted with a cell homogenizer (Braun, Melsungen, Germany) as described (6), and the homogenate was centrifuged at 4° and 6000 × *g* for 15 min. The resulting supernatant (crude extract) was assayed for acyl-CoA synthetase activity at 25° by the hydroxamic acid method. The assay procedure was the same as described

Table 1. Acyl-CoA synthetase activity in cells grown on glucose or oleic acid

Strain	Specific activity (nmol/min per mg of protein)	
	Glucose-grown cells	Oleic acid-grown cells
Y-6795	60.7	41.6
L-5	0.7	2.8
L-7	0.7	2.7
RL7-1	74.6	50.4
RL7-2	63.8	46.3
RL7-8	60.0	45.2

Cells were grown in B medium containing either 2% glucose or 1% oleic acid and were harvested at the late-logarithmic phase. Crude extracts from the cells were assayed for enzyme activity.

by Pande and Mead (7), except that 0.04% Triton X-100 (Rohm and Haas, Philadelphia, PA) was added to the assay mixture and that after the reaction 1.5 mg of bovine serum albumin (Sigma) was added to coprecipitate long-chain acyl hydroxamic acid; 4 mM potassium oleate and 0.6 mM CoA were used as substrates.

[^{14}C]Oleate Incorporation Studies. Approximately 10^7 cells were inoculated into 25 ml of NB medium containing 50 μCi of [*1*-^{14}C]oleic acid (0.62 mCi/mmol, Radiochemical Centre, Amersham, England) or 25 μCi of [*10*-^{14}C]oleic acid (0.31 mCi/mmol, Commissariat a l'Énergie Atomique, Saclay, France) and were grown for 18 hr to yield 0.7 to 1 × 10^9 cells. The cells were washed four times with SB solution containing 0.1% oleic acid, four times with SB solution, and once with water. The cells, suspended in water, were heated in a boiling-water bath for 2 min and then washed three times with SB solution and once with water. Cellular lipids were extracted from the washed cells as described (1), except that carrier cells were not added. An aliquot (about 3 × 10^5 cpm) of the radioactive lipids was subjected to thin-layer chromatography under the conditions described (1). The polar lipid and triglyceride, eluted with chloroform/methanol 2:1, were saponified with 20% methanolic KOH at 80° for 3 hr. The fatty acids liberated were isolated, and the radioactivity present in the carboxyl carbon of the fatty acids was determined by the Sehmidt reaction essentially as described by Brady *et al.* (8); [*U*-^{14}C]palmitic acid (Radiochemical Centre), [*1*-^{14}C]oleic acid, and [*10*-^{14}C]oleic acid were used as authentic standards. Radioactivity was determined with the scintillator solution of Patterson and Greene (9) in a liquid scintillation spectrometer.

Analytical Methods. The composition of cellular fatty acids was analyzed by gas-liquid chromatography as described (10). Protein was determined by the method of Lowry *et al.* (11), with bovine serum albumin as the standard.

RESULTS

Isolation and Properties of Mutants. Mutant strains of *C. lipolytica* defective in acyl-CoA synthetase were selected on the basis of the rationale that acyl-CoA synthetase activity would become indispensable when yeast cells in which fatty acid synthesis *de novo* is blocked by cerulenin are grown in a medium supplemented with fatty acid (1). Among the 19 mutant strains isolated at an overall rate of 10^{-5} to 10^{-6}, two strains, L-5 and L-7, were studied extensively.

Eight revertant strains were isolated from the mutant strain L-7 with a frequency of 5 × 10^{-7} to 2 × 10^{-10}, while no revertant was found among 1.5 × 10^{11} cells of the strain L-5. All

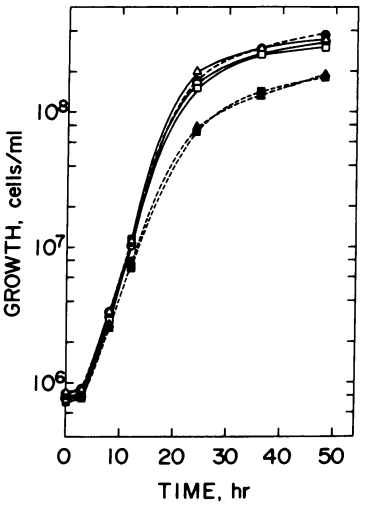

FIG. 1. Growth curves for the wild-type and mutant strains. About 4 × 10^6 cells were inoculated into 5 ml of NB medium containing either 2% glucose or 1% oleic acid and were incubated with agitation at 175 reciprocations per min. Viable cells were counted on YPG agar plates. ○, Y-6795 grown on glucose; ●, Y-6795 grown on oleic acid; △, L-5 grown on glucose; ▲, L-5 grown on oleic acid; □, L-7 grown on glucose; ■, L-7 grown on oleic acid.

revertant strains were capable of growing in the presence of cerulenin and fatty acid at rates comparable to that observed for the wild-type strain. Three strains, RL7-1, RL7-2, and RL7-8, were examined in detail.

It is evident from Table 1 that the crude extracts from the mutant cells grown on glucose or oleic acid exhibited very little acyl-CoA synthetase activity under the assay conditions used. The revertant cells exhibited acyl-CoA synthetase activity comparable to that of the wild-type cells.

Fig. 1 represents the growth curves for the wild-type and mutant strains. The mutant strains grew on oleic acid as a sole carbon source almost as fast as the wild-type strain did. *n*-Pentadecane also supported the growth of the mutant strains. This was unexpected in view of the finding that the mutant cells exhibited very little acyl-CoA synthetase activity *in vitro* and that they failed to grow in the presence of cerulenin and fatty acid. The following studies were designed to understand the mechanism by which fatty acid is metabolized in the mutant cells to support their growth.

Composition of Cellular Fatty Acids of Mutants. The cellular fatty acids of the wild-type strain of *C. lipolytica* are principally of odd-numbered chain lengths when cells are grown on an odd-chain-length *n*-alkane such as *n*-pentadecane or *n*-heptadecane (10). In the experiments presented in Table 2, the wild-type, mutant, and revertant strains were grown on an odd-chain-length fatty acid or *n*-alkane and the composition of cellular fatty acids was analyzed. The mutant cells contained mainly even-chain-length fatty acids, whereas the fatty acids derived from the wild-type and revertant cells were principally of odd-numbered chain lengths. Control cells of all strains grown on even-chain-length fatty acid or *n*-alkane contained preferentially even-chain-length fatty acids. These results indicate that the mutant strains, unlike the wild-type and revertant strains, cannot incorporate exogenous fatty acid or the fatty acid derived from *n*-alkane as a whole into cellular lipids, but utilize the fatty acid that is synthesized *de novo* from acetyl-CoA produced by degradation of the carbon source. Thus, it was assumed that in the mutant strains exogenous fatty acid would be converted to such an activated form or be activated in such a cellular compartment that the activated derivative

Table 2. Relative content of odd-chain-length fatty acids in cells grown on fatty acid or *n*-alkane

Strain	Odd-chain-length fatty acids in cells grown on, %*			
	Pentadecanoic acid	Tetradecanoic acid	*n*-Heptadecane	*n*-Octadecane
Y-6795	94.7	2.3	98.5	3.0
L-5	6.1	0.6	14.3	2.6
L-7	5.8	0.4	28.3	2.6
RL7-1	91.7	2.5	—	—
RL7-8	93.2	1.4	—	—

Cells were grown in B medium containing 1% Brij 58 and either 0.5% fatty acid or 0.8% *n*-alkane. The cells were harvested at the stationary phase and washed three times with 4% Triton X-100 and once with water. The composition of cellular fatty acids was determined.

* The sum of the amounts of the fatty acids detected is taken as 100%. The compositions of the cellular fatty acids of the wild-type and revertant strains grown on any of the carbon sources used as well as of the mutant strains grown on tetradecanoic acid or *n*-octadecane were essentially the same as that of the wild-type strain grown on *n*-alkane of the corresponding chain length (10). The relative contents of major fatty acids in the mutant strains grown on the carbon sources of odd-numbered chain lengths were as follows. L-5 grown on pentadecanoic acid: 18:1, 37%; 18:2, 24%; 16:1, 21%; 16:0, 11%. L-7 grown on pentadecanoic acid: 18:1, 35%; 18:2, 26%; 16:1, 20%; 16:0, 12%. L-5 grown on *n*-heptadecane: 18:2, 42%; 18:1, 15%; 16:1, 15%; 16:0, 13%; 17:1, 6%; 17:0, 4%. L-7 grown on *n*-heptadecane: 18:2, 31%; 16:1, 14%; 17:0, 14%; 18:1, 13%; 16:0, 11%; 17:1, 8%; 17:2, 4%.

would be degraded to yield acetyl-CoA but not utilized for the synthesis of cellular lipids.

Activation of Fatty Acid in Mutants. On the basis of the results just described, we next studied the mechanism involved in the activation of exogenous fatty acid in the mutants. Fatty acid could be activated to its CoA derivative either by a nucleoside triphosphate-dependent ligase reaction or by an NAD^+-dependent aldehyde dehydrogenase reaction. Hence, the following four mechanisms were considered: first, thioesterification of fatty acid catalyzed by an additional acyl-CoA synthetase that has not been detected yet; second, ω-oxidation of fatty acid followed by thioesterification of the resulting dicarboxylic acid; third, ω-oxidation of fatty acid followed by CoA-dependent dehydrogenation of the resulting semialdehyde; fourth, α-oxidation of fatty acid followed by CoA-dependent oxidative decarboxylation of the resulting α-keto acid.

In order to determine which mechanism is valid, we cultivated cells for six to seven generations with [*1*-^{14}C]oleic acid or [*10*-^{14}C]oleic acid as a sole carbon source and then determined the incorporation of radioactivity into cellular lipids as well as the ^{14}C content of the carboxyl carbon relative to that of the total carbons of the fatty acids liberated from the lipids. The following results would be expected for each of the four mechanisms mentioned above. The first mechanism would predict that the ^{14}C derived from [*1*-^{14}C]oleic acid is equally distributed among the carbon atoms of odd numbers of the cellular fatty acids, while the ^{14}C derived from [*10*-^{14}C]oleic acid is equally distributed among the carbon atoms of even numbers of the fatty acids. The second mechanism, in conjunction with the assumption that the dicarboxylic acid is activated at either carboxyl group, would predict that the ^{14}C derived from [*1*-^{14}C]oleic acid labels the carbon atoms of odd numbers of the cellular fatty acids to an equal extent, while the ^{14}C derived from [*10*-^{14}C]oleic acid is uniformly distributed in all carbon atoms of the fatty acids. Both the third and the fourth mechanism would predict that the ^{14}C derived from [*1*-^{14}C]oleic acid is not incorporated into the cellular fatty acids, while the ^{14}C derived from [*10*-^{14}C]oleic acid is equally distributed among the carbon atoms of odd numbers of the fatty acids.

Table 3. Incorporation of ^{14}C into fatty acyl moiety of polar lipid and triglyceride from cells grown on [*1*-^{14}C]oleic acid or [*10*-^{14}C]-oleic acid and ratio of ^{14}C content of total carbons to that of carboxyl carbon of the fatty acids

Strain	[*1*-^{14}C]Oleic acid		[*10*-^{14}C]Oleic acid	
	Polar lipid	Triglyceride	Polar lipid	Triglyceride
	Incorporation, cpm × 10^{-5}*			
Y-6795	21.9	103.0	13.0	32.0
L-5	14.6	4.8	11.3	3.8
L-7	32.0	9.9	17.7	8.3
RL7-1	48.3	105.1	—	—
RL7-8	43.3	146.3	—	—
	Total carbons/carboxyl carbon†			
Y-6795	0.99	1.01	875	575
L-5	8.25	7.69	214	205
L-7	7.22	6.82	301	264
RL7-1	1.00	1.04	—	—
RL7-8	1.01	1.02	—	—

* Values are expressed as the radioactivity incorporated into the fatty acyl moiety of each lipid class derived from 10^9 cells. The ratio of the radioactivity in the fatty acyl moiety to that in the whole lipid molecule was, regardless of the strain used, 0.86–0.96 for the polar lipid from cells grown on [*1*-^{14}C]oleic acid, 0.75–0.80 for the polar lipid from cells grown on [*10*-^{14}C]oleic acid, and 0.92–0.96 for the triglyceride from cells grown on either [*1*-^{14}C]oleic acid or [*10*-^{14}C]oleic acid.

† Aliquots of the fatty acids (10,000–30,000 cpm) were subjected to decarboxylation by the Schmidt reaction. Values for authentic [*U*-^{14}C]palmitic acid, [*1*-^{14}C]oleic acid, and [*10*-^{14}C]oleic acid were 16.2, 1.02, and 165, respectively.

Table 3 represents the results of the isotope incorporation studies actually obtained. Regardless of which [^{14}C]oleic acid was used as the carbon source, comparable amounts of radioactivity were incorporated into the fatty acyl moiety of the polar lipid as well as the triglyceride isolated from the mutant cells; this holds even if the difference between the specific radioactivity of [*1*-^{14}C]oleic acid and that of [*10*-^{14}C]oleic acid is taken into consideration (see *Materials and Methods*). Hence, the third and fourth mechanisms are excluded. This is further supported by the finding that essentially no radioactivity was found in the carboxyl carbon of the fatty acids derived from the mutant cells grown on [*10*-^{14}C]oleic acid. This finding excludes the second mechanism as well and supports the first mechanism. Consistent with the first mechanism is also the finding that one-seventh to one-eighth of the total radioactivity present in the fatty acids derived from the mutant cells grown on [*1*-^{14}C]oleic acid was found in the carboxyl carbon. Thus, it is concluded that in the mutant cells exogenous fatty acid is activated by an additional acyl-CoA synthetase. We designate the enzyme lacking in the mutant strains as acyl-CoA synthetase I. The data obtained with the wild-type and revertant cells are consistent with the direct incorporation of exogenous fatty acid as a whole into cellular lipids.

The mutant cells incorporated much smaller amounts of ^{14}C derived from labeled oleic acid into triglyceride than did the wild-type and revertant cells, while similar amounts of radioactivity were incorporated into polar lipid in all strains. Since the ratio of the radioactivity in the fatty acyl moiety to that in

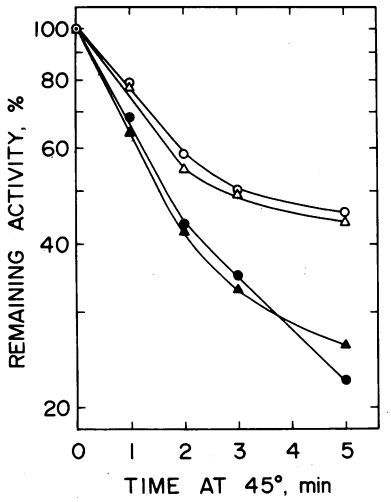

FIG. 2. Thermosensitivity of acyl-CoA synthetase I from the wild-type and revertant strains. Cells were grown in B medium containing 2% glucose and were harvested at the late-logarithmic phase. Aliquots (approximately 0.3 mg) of crude extracts from the cells were preincubated at 45° in the assay mixture devoid of ATP and CoA for the lengths of time indicated. The reaction was then initiated by the addition of ATP and CoA and was allowed to proceed at 25°. The enzyme activities measured without preincubation were 68.0, 76.1, 69.1, and 65.0 nmol/min per mg of protein for Y-6795 (○), RL7-1 (△), RL7-2 (●), and RL7-8 (▲), respectively.

the whole triglyceride molecule was almost the same in all strains, it can be concluded that the observed difference in the radioactivity incorporated into triglyceride actually reflects the difference in the amount of triglyceride synthesized.

Thermosensitivity of Acyl-CoA Synthetase I from Revertants. From the results presented in Tables 1–3, it is evident that the phenotypes of the revertant strains, which were isolated without mutagenesis with a reasonably high frequency, are indistinguishable from those of the wild-type strain. This indicates that the characteristics of the mutant strains are due to a single mutation which results in the lack of acyl-CoA synthetase I. The experiment shown in Fig. 2 was designed to examine whether this mutation is located in the structural gene of the enzyme. The results obtained showed that the acyl-CoA synthetase I of the revertant strains RL7-2 and RL7-8 was more thermosensitive under the conditions used than that of the wild-type strain and the revertant strain RL7-1. This suggests that the mutant strain L-7 carries a mutation in the structural gene of acyl-CoA synthetase I.

DISCUSSION

The present investigation with the use of the mutant strains has disclosed a characteristic feature of fatty acid metabolism in *C. lipolytica*. This organism must possess at least two distinct acyl-CoA synthetases that activate long-chain fatty acid. One, designated acyl-CoA synthetase I, which is absent in the mutant strains, is responsible for the production of acyl-CoA to be utilized for the synthesis of cellular lipids. The other acyl-CoA synthetase provides an acyl-CoA that is exclusively degraded via β-oxidation to yield acetyl-CoA. In fact, we have obtained evidence indicating the presence of a second acyl-CoA synthetase both in the wild-type and in the mutant cells grown on fatty acid and have been able to separate this enzyme from acyl-CoA synthetase I (M. Mishina, T. Kamiryo, S. Tashiro, and S. Numa, unpublished data). This strengthens our conclusion that the mutant strains are defective in acyl-CoA synthetase I.

The mutant strains synthesize much smaller amounts of triglyceride than do the wild-type and revertant strains. This finding suggests that the observed decrease in triglyceride synthesis results from the lack of acyl-CoA synthetase I.

C. lipolytica grown on hydrocarbons is one of the most prominent sources of single-cell protein (12). Since the mutant strains of this organism defective in acyl-CoA synthetase I synthesize cellular lipids containing even-chain-length fatty acids from odd-chain-length hydrocarbons, the use of these mutants for the production of single-cell protein appears to be of great advantage from a nutritional point of view.

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