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Biochemistry

Fatty acid synthetase from *Brevibacterium ammoniagenes*: Formation of monounsaturated fatty acids by a multienzyme complex

(3-decynoyl-*N*-acetylcysteamine/β,γ-dehydration of β-hydroxyacyl thioester)

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ABSTRACT **A multienzyme fatty acid synthetase complex isolated from *Brevibacterium ammoniagenes* has been purified to a specific activity of 1440 nmol of malonyl-CoA incorporated per min/mg. The enzyme is homogeneous, as judged by gel electrophoresis on agarose gels, and has a molecular weight of 1.2×10^6. Both NADPH and NADH are required for activity. In contrast to other fatty acid synthetase complexes, the enzyme catalyzes the synthesis of both long-chain saturated and monounsaturated fatty acids from malonyl-CoA and acetyl-CoA. The formation of unsaturated fatty acids is oxygen-independent and sharply reduced by 3-decynoyl-*N*-acetylcysteamine, a known inhibitor of *Escherichia coli* β-hydroxydecanoyl thioester dehydrase (EC 4.2.1.60).**

Multienzyme fatty acid synthetase complexes have been isolated from various eukaryotic sources (1). By contrast, fatty acid synthesis in plants and most bacteria is catalyzed by individual, nonaggregating enzymes (1). Exceptions to this generalization have been reported for some more advanced prokaryotes. *Mycobacterium smegmatis* and *Corynebacterium diphtheriae* use multienzyme complexes for long-chain fatty acid synthesis (2, 3).

We now describe the purification of a multienzyme complex from the bacterium *Brevibacterium ammoniagenes*. This enzyme system catalyzes the synthesis of monounsaturated as well as saturated fatty acids from malonyl-CoA and acetyl-CoA. This property has not been previously observed for multienzyme complexes of this type.

MATERIALS AND METHODS

Materials. Malonyl-CoA and acetyl-CoA were obtained from Sigma; [2-^{14}C]malonyl-CoA (32.8 μCi/μmol) was from New England Nuclear; β-cyclodextrin was from Aldrich Chemical Co.; NADPH and NADH were from Oriental Yeast Co. (Japan). Heptakis(2,6-di-*O*-methyl)β-cyclodextrin was prepared by the procedure of Bergeron *et al.* (4). All other chemicals were of analytical grade.

Bacteria. *Brevibacterium ammoniagenes* IAM 1641 (ATCC 6871) cells were grown to early stationary phase in a medium containing (wt/vol): meat extracts (0.7%), peptone (1.0%), NaCl (0.3%), and dextrose (1.0%).

Enzyme Assay. Fatty acid synthetase activity was assayed either by incorporation of radioactive substrate or spectrophotometrically. Unless otherwise stated, assays were carried out at 37° in mixtures containing, in a total volume of 0.5 ml: 0.4 M potassium phosphate buffer (pH 7.3), 5 mM dithiothreitol, 100 μM NADPH, 100 μM NADH, 40 μM malonyl-CoA, 50 μM acetyl-CoA, 50 μg of heptakis(2,6-di-*O*-methyl)β-cyclodextrin, and enzyme protein. For radioactive assays (5), [2-^{14}C]malonyl-CoA (56,200 dpm) was used. Spectrophotometric assays were performed according to Lynen (6). One unit of activity is defined as the amount of enzyme required to incorporate 1 nmol of malonyl-CoA per min into fatty acids.

Identification of Fatty Acids Synthesized. Radioactive fatty acids synthesized from [2-^{14}C]malonyl-CoA were methylated by diazomethane after the addition of carrier fatty acids (100 μg each of palmitic, stearic, and oleic acids). The methyl esters were analyzed by gas–liquid chromatography on a 1.5-m column of 1.5% SE-30 on Chromosorb W (AW-DMCS). Effluent was burned to CO_2 and water and passed through a continuous anthracene scintillation counter (Shimadzu RID-2E, Japan).

Purification of Fatty Acid Synthetase. All steps were performed at 0–4°. Protein was determined by the method of Schaffner and Weissmann (7). All buffers contained 10 mM 2-mercaptoethanol and 1 mM EDTA, unless otherwise stated.

Fifty grams of frozen *B. ammoniagenes* cells were thawed in 50 ml of 0.1 M potassium phosphate buffer (pH 7.0). The cells were broken with 200 g of glass beads (0.17–0.18 mm) in a Waring Blendor for a total of 15 min in 5-min periods with 5-min intervals. After addition of a 50-ml portion of buffer with stirring, the disrupted cells were centrifuged at 25,000 × *g* for 20 min. The resulting supernatant was treated with 0.1 mg of deoxyribonuclease (beef pancreas, 810 units/mg, Miles Laboratories, Inc.) for 10 min. The deoxyribonuclease-treated supernatant was diluted 1:5 by 0.05 M potassium phosphate buffer (pH 7.0), and DEAE-cellulose (50 g of Whatman DE-52), which had been previously equilibrated with the same buffer, was then added. After the mixture was stirred for 30 min, DEAE-cellulose containing absorbed enzyme was filtered and washed with 500 ml of 0.25 M potassium phosphate buffer (pH 7.0) for 30 min with stirring. After filtration, the DEAE-cellulose was suspended in 100 ml of 0.25 M potassium phosphate buffer (pH 7.0) and packed in a column. The enzyme was eluted with 0.5 M potassium phosphate buffer (pH 7.0). The major fractions containing enzyme activity were brought to 60% saturation with ammonium sulfate and centrifuged at 25,000 × *g* for 20 min. The precipitate was dissolved in 5 ml of 0.5 M potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1 mM dithiothreitol instead of 10 mM 2-mercaptoethanol, and applied to a Sepharose 6B column (3.1 × 67 cm) that had been equilibrated with the same buffer. The fractions containing activity were combined and concentrated on a Diaflo apparatus with XM-50 membrane. The results of this purification are summarized in Table 1. After the final purification step the synthetase was homogeneous, as judged by the appearance of a

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Table 1. Purification of fatty acid synthetase from *B. ammoniagenes*

Fraction	Volume, ml	Total activity, units	Total protein, mg	Specific activity, units/mg	Yield, %
Crude extracts	150	18,500	1350	13.7	100
DEAE-cellulose	70	14,700	108	136	79.4
Sepharose 6B*	15	6,160	4.28	1440	33.3

* This fraction showed a specific activity of 1100 units/mg when assayed without heptakis(2,6-di-*O*-methyl)β-cyclodextrin.

single band when subjected to electrophoresis on 1% agarose gels (Fig. 1).

RESULTS

Properties of Fatty Acid Synthetase. The pH optimum of the enzyme activity was 7.3. Both NADPH and NADH were required for enzyme activity; relatively high concentrations of potassium phosphate buffer (optimal at 0.4 M phosphate) were also required. In each elongation cycle of fatty acid synthesis, reduced pyridine nucleotides are consumed in two separate steps—the reduction of β-ketoacyl and of α,β-enoyl derivatives, respectively. It is not possible to determine the respective nucleotide specificities directly, but information obtained with the fatty acid synthetase complex from *M. smegmatis* indicates NADPH-specific β-ketoacyl and NADH-specific α,β-enoyl reduction at least in bacterial systems (8). In agreement with these observations, we have found for the *Brevibacterium* system incorporation of deuterium into fatty acids from either deuterio-NADPH or deuterio-NADH in the presence of NADH or NADPH, respectively (9).

The K_m values for substrates were: malonyl-CoA, 9 μM; acetyl-CoA, 11 μM; NADPH, 18 μM; and NADH, 11 μM. The molecular weight, estimated by chromatography on Sepharose 6B, was 1.2×10^6 in three trials.

Identification of Products. In order to determine whether the synthetase products are free or esterified fatty acids, an enzyme reaction was carried out with [2-^{14}C]malonyl-CoA for 15 min and stopped by the addition of 0.075 ml of 12% perchloric acid. Almost all of the ^{14}C-labeled products were extractable by butanol. The butanol extracts were chromatographed on cellulose thin-layer plates with ethanol/acetate buffer (pH 4.5) (7:3) as the solvent. More than 90% of the radioactivity was located in the zone of fatty acyl-CoA derivatives. This result suggested that the products of the fatty acid synthetase were CoA derivatives.

Fig. 2 shows the composition of radioactive fatty acids synthesized from [2-^{14}C]malonyl-CoA. Radioactivity is distributed among palmitate, octadecenoate, and stearate (19:46:35, respectively). The peak corresponding to an octadecenoic acid was identified as oleic acid as follows. Methyl octadecenoate was subjected to permanganate/periodate oxidation (10). The methyl esters of the resulting monocarboxylic and dicarboxylic acids were analyzed by gas–liquid chromatography (Fig. 3). The association of radioactivity with the methyl esters of pelargonic acid and azelaic acid identifies the unsaturated fatty acid as 9,10-octadecenoic acid. We have not determined the configuration of the radioactive octadecenoic acid but assume it to be *cis*, since the constituent unsaturated fatty acid of *Brevibacterium* cells was identified as oleic acid.

Time Course of Fatty Acid Synthesis. Fig. 4 shows the time course for the synthesis of saturated and unsaturated fatty acids. Saturated and unsaturated acids are formed at a linear rate during the first 15 min of incubation. Moreover, there are no significant differences in the ratio of saturated and unsaturated acids throughout a 50-min incubation period. This constant ratio suggests that the fatty acid synthetase introduces the double bond during the process of chain elongation rather than by desaturation of preformed long-chain saturated fatty acids. It has also been observed that the synthesis of saturated and unsaturated fatty acids declines with identical rates when enzyme solutions are heated to 50°.

Effects of Atmosphere and Inhibitor on Unsaturated Fatty Acid Synthesis. The results described above suggested that the *Brevibacterium* fatty acid synthetase complex produces unsaturated and saturated fatty acids. Two distinct biochemical pathways have been recognized for the introduction of double bonds (11). In one of the two pathways, long-chain fatty acyl-CoA derivatives are desaturated to the corresponding *cis*-Δ^9-monoenoic acids by particle-bound enzymes that require molecular oxygen for activity. A second pathway proceeds anaerobically, introducing a *cis*-3 double bond into a medium-chain-length fatty acid (usually C_{10}) by β,γ-dehydration of a

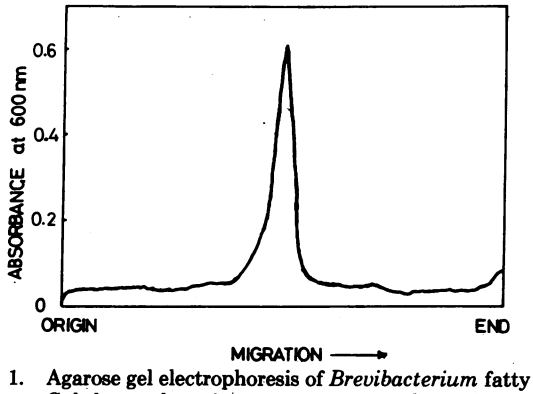

FIG. 1. Agarose gel electrophoresis of *Brevibacterium* fatty acid synthetase. Gel electrophoresis on 1% agarose was done according to the methods of Holden *et al.* (16). After the gels were destained they were scanned on a Fujiox (Tokyo, Japan) densitometer.

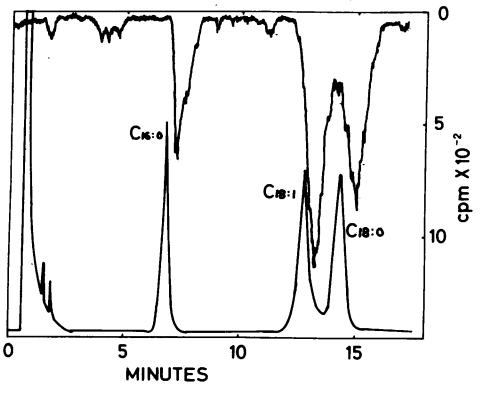

FIG. 2. Gas–liquid chromatography of methyl esters of fatty acids produced by the fatty acid synthetase. Experimental details are described in *Materials and Methods*. The column temperature was 180°. A conventional mass record and radioactivity tracings are shown.

Table 2. Effects of atmosphere and 3-decynoyl-*N*-acetylcysteamine on fatty acid synthesis

Exp.	Atmosphere	3-Decynoyl-*N*-acetylcysteamine, M	Fatty acids, cpm		Saturated/unsaturated
			Saturated	Unsaturated	
1	Argon	0	4140	4690	0.88
	Argon	0	5030	5890	0.85
	Air	0	5020	5790	0.87
	Air	0	4240	4770	0.89
2	Air	0	5190	5870	0.88
	Air	10^{-6}	5720	3930	1.46
	Air	10^{-5}	5740	2310	2.48
	Air	10^{-4}	5620	990	5.68

Experimental details are described in the legend of Fig. 4. 3-Decynoyl-*N*-acetylcysteamine was dissolved in the reaction mixtures according to the methods of Norris *et al.* (15). In Exp. 2, enzyme was incubated for 5 min at 37° with 3-decynoyl-*N*-acetylcysteamine before the reaction was started.

fatty acid synthesis intermediate, β-hydroxyacyl thioester. Chain elongation of the resulting 3-enoates gives rise to long-chain unsaturated fatty acids. This dehydration is catalyzed by a specific enzyme, β-hydroxydecanoyl thioester dehydrase (EC 4.2.1.60), which has been purified from *Escherichia coli* (12). Several experiments were carried out to determine which of the two pathways is catalyzed by the fatty acid synthetase of *B. ammoniagenes*. When enzyme reactions were carried out in an argon atmosphere, radioactivity incorporated into saturated and unsaturated fatty acids from [2-^{14}C]malonyl-CoA was almost the same as in the presence of air (Table 2, Exp. 1). Thus, the synthesis of the unsaturated acid did not require molecular oxygen. In another series of experiments we measured the effects of 3-decynoyl-*N*-acetylcysteamine on the synthesis of unsaturated fatty acids (Table 2, Exp. 2). This compound is a powerful inhibitor of β-hydroxydecanoyl thioester dehydrase from *E. coli* (13) and consequently abolishes specifically the synthesis of unsaturated fatty acids by the *E. coli* synthetase (14). This acetylenic compound also had inhibitory effects on the unsaturated fatty acid synthesis by the *B. ammoniagenes* synthetase, but did not affect the formation of saturated fatty acids.

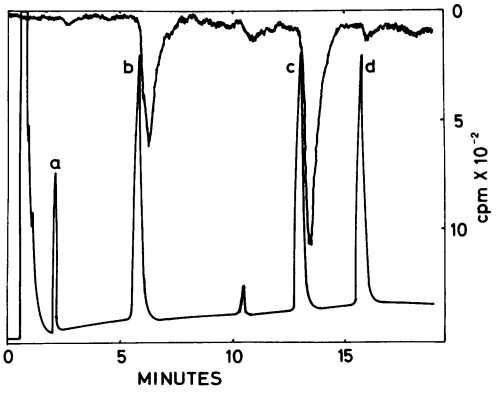

FIG. 3. Gas–liquid chromatography of methyl esters of oxidation products of octadecenoic acid. The radioactive fatty acids made from [2-^{14}C]malonyl-CoA were methylated by diazomethane after the addition of oleic and *cis*-vaccenic acids (100 μg each). Methyl esters of unsaturated fatty acids were separated from those of saturated fatty acids on a $AgNO_3$-impregnated silica gel thin-layer plate and were subjected to permanganate/periodate oxidation (10). Products extracted with ether were methylated with diazomethane and analyzed by gas–liquid chromatography on a 1.5% SE-30 column. The column temperature was maintained at 100° for the first 5 min and then programmed from 100° to 200° at 10° per min. (*a*) Heptanoic acid; (*b*) pelargonic acid; (*c*) azelaic acid; (*d*) 1,11-undecanedioic acid.

DISCUSSION

The present paper describes the purification of a multienzyme fatty acid synthetase complex from *B. ammoniagenes*. The novel feature of this enzyme complex is its ability to synthesize not only saturated fatty acids, but also an unsaturated fatty acid. Every multienzyme complex previously isolated synthesizes saturated fatty acids exclusively (1). Several lines of evidence suggest that the *Brevibacterium* system synthesizes the unsaturated fatty acid by an anaerobic process involving β,γ-dehydration of β-hydroxyacyl thioester intermediates and subsequent chain elongation of β,γ-enoate without reduction of the double bond. Similar results have already been reported for the fatty acid synthetase system of *E. coli* (14). However, the *B. ammoniagenes* synthetase and the synthetase system from *E. coli* are not of the same type. Fatty acid synthesis in *E. coli* is catalyzed by individual, nonaggregating enzymes, whereas the synthetase from *B. ammoniagenes* appears to be a tightly aggregated multienzyme complex. Second, the enzymes of *E. coli* produce predominantly *cis*-vaccenic acid (Δ^{11}-C_{18}) with the aid of a β-hydroxydecanoyl thioester dehydrase. On the other hand, the *B. ammoniagenes* system produces oleic acid (Δ^{9}-C_{18}) exclusively. Since neither Δ^{11}-C_{18} nor Δ^{9}-C_{16} acids were

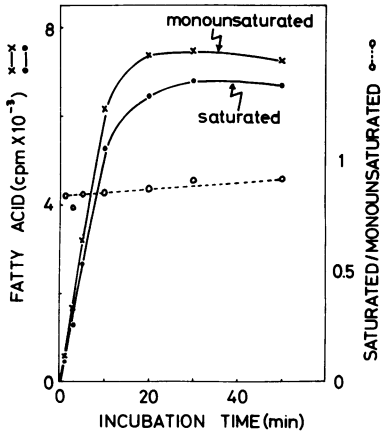

FIG. 4. Time course of fatty acid synthesis. The methyl esters of radioactive fatty acids formed from [2-^{14}C]malonyl-CoA (56,200 dpm) were separated into saturated and unsaturated fractions by thin-layer chromatography on $AgNO_3$-impregnated silica gel, with *n*-hexane/diethyl ether (9:1) as the solvent. The fatty acid ester spots were visualized under ultraviolet light after the plate was sprayed with 2′,7′-dichlorofluorescein (0.2% in ethanol). The spots were scraped from the plate and radioactivity was determined directly in toluene scintillation solution in a Beckman LS-230 liquid scintillation spectrometer.

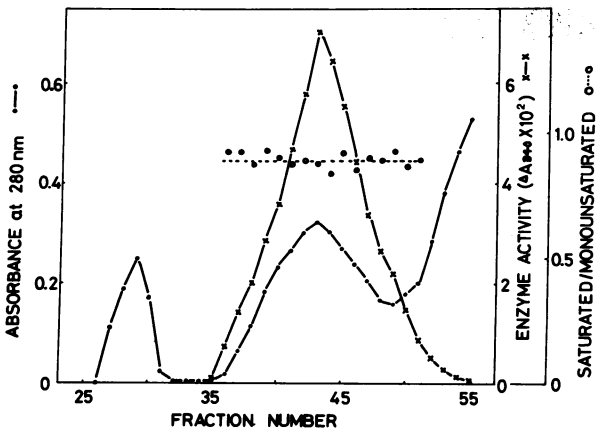

FIG. 5. Elution profile of *Brevibacterium* fatty acid synthetase from a Sepharose 6B column. DEAE-cellulose fraction in Table 1 was applied to a Sepharose 6B column (details are described in *Materials and Methods*). The synthesis of saturated and monounsaturated fatty acids is determined by the methods described in the legend of Fig. 4. The ratio of saturated to monounsaturated fatty acids is shown (O - - - O).

detectable among the products, dehydration leading to β,γ-enoate should occur at the C_{12} level specifically. It is noteworthy that the *B. ammoniagenes* synthetase is approximately two orders of magnitude less sensitive to 3-decynoyl-*N*-acetylcysteamine inhibition than the *E. coli* synthetase system (compare the data in Table 2 with those in ref. 13). The dehydrase of *E. coli* exhibits a strong preference for both C_{10} thioester substrates and C_{10} acetylenic inhibitor. A coincident chain length specificity for substrate and inhibitor is also likely for the *B. ammoniagenes* synthetase. Since the postulated dehydrase activity of *B. ammoniagenes* would be expected to have C_{12} substrate specificity, 3-dodecynoyl-*N*-acetylcysteamine might be a more effective inhibitor in this case.

The ratio of saturated to unsaturated fatty acids was essentially constant across the enzyme activity peak on Sepharose 6B profile (Fig. 5) and also constant for all preparations throughout enzyme purification. For these reasons and because the complex appears to be homogeneous, we are inclined to the view that the enzyme component responsible for oleate formation is tightly associated with the various activities for carbon chain elongation as an integral part of the *Brevibacterium* fatty acid synthetase complex.

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