# IDENTIFICATION OF A MULTIENZYME COMPLEX SYNTHESIZING FATTY ACIDS IN THE ACTINOMYCETE *Streptomyces erythreus*

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Received December 13,1972

*Summary*

The streptomycetes have a bacterial-type cell wall and a mycelial growth habit. Although the exact taxonomic relationship to yeasts or fungi and the bacteria is unknown, a great similarity to bacteria is assumed. *Streptomyces erythreus* CA340 (Abbott) is found to possess a multienzyme complex making fatty acids. The crude enzyme complex is stimulated by FMN; is inhibited by iodoacetamide; is excluded and partially purified (ca. 70-fold) by Sephadex G-200; is most active at pH 7.5; is most stable in 0.5 M phosphate buffer. Thus, *S. erythreus* CA340 and possibly all streptomycetes have a major biochemical similarity to yeasts and fungi.

## *Introduction*

Members of the genus streptomyces of the order actinomycetales grow in a mycelial habit and are genetically much more complex than bacteria (1). Classified as the *fungi imperfecti* they have nonetheless been considered to be closely related to the bacteria. For instance, their mycelia and spores have a diameter that is similar to that of bacteria and their cell walls lack chitin and are sensitive to lysozyme. However, taxonomic classification would be more reliable if it were based on biochemical as well as morphological characteristics. One such distinctive characteristic may be the fatty acid synthetase enzyme system. Yeasts, fungi and higher forms of life employ a non-dissociable multienzyme complex (type I) to synthesize their fatty acids, whereas bacteria carry out the same synthesis with dissociable enzymes (Type II) (1). Both synthetases require a pantetheine moiety bound either to the multienzyme complex (I) or to a separate small carrier protein (II) (2,3). Thus, definition of the nature of fatty acid biosynthesis by a

streptomycete should give valuable information about its evolutionary status.

*Streptomyces erythreus* CA340 (Abbott) is the producer of large amounts of the antibiotic erythromycin, and some time ago fatty acid synthetase activity was implicated in crude cell-free extracts derived from it (4). The instability of the fatty acid synthetase activity frustrated further efforts to characterize it and an in vivo incorporation of a radioactive label was therefore attempted (5). Results from experiments utilizing tritiated ß-alanine, a part of the pantetheine prosthetic group, indicated the existence of a very high (>$10^6$ daltons from Sepharose 6B chromatography) as well as a low (<$3.0 \times 10^4$ daltons) molecular weight protein fraction (6). This suggested the possibility that fatty acid synthetases of both type I and type II are present in *S. erythreus* CA340. In this communication we wish to confirm the presence of a multienzyme complex fatty acid synthetase in this organism. It is of high molecular weight, forms a fatty acid mixture including palmitate and requires FMN for optimal activity.

## *Materials and Methods*

The strain of *Streptomyces erythreus* CA340 used was a gift of Abbott Laboratories. Its maintenance and growth conditions have been described (7). Fatty acid synthetase activity is dependent on the stage of growth of the organism and cultures were collected after 12 hours and extracts prepared as described (4). The boiled extract supernatant was prepared by heating the extract to 100°C for 10 minutes and centrifuging to remove precipitated protein.

An acetone precipitation of the extract was carried out by adding the crude extract to 10 volumes of stirred, pre-chilled (-30°C) acetone. After 15 minutes, the precipitated solids were collected by filtration and washed once with 5 volumes of chilled acetone. The powder was thoroughly dried under vacuum at 0°C in a desiccator. For the assays, the acetone powder was

homogenized (200 mg/ml) in 0.5 M potassium phosphate buffer, pH 7.0,containing $10^{-3}$M DTT and $10^{-3}$M EDTA.

Reagents were of analytical reagent grade. 1-$^{14}$C-acetyl CoA and 1,3-$^{14}$C-malonyl CoA were purchased from New England Nuclear and diluted with cold material from P. L. Biochemicals. Flavine mononucleotide (FMN) and NADPH were obtained from Sigma. The Sephadex G-50 and G-200 were purchased from Pharmacia Fine Chemicals.

Radioactivity was measured in a Nuclear Chicago Mark I scintillation counter in scintillation fluid composed of 100 g napthalene, 5 g of 2,5-diphenyloxazole, 0.3 g of 1,4-bis-2(4 methyl-5 phenyl oxazolyl)-benzene, 730 ml 1,4-dioxane, 135 ml toluene, and 35 ml methanol. The efficiency obtained was 80%. Protein was determined by the method of Lowry (8).

## *Results and Discussion*

Although readily demonstrated, fatty acid synthesis in extracts of *S. erythreus* CA340 was weak, capricious and unstable (4). The major product as identified by TLC (9) is palmitate. Attempts at purification were generally unsuccessful, although ammonium sulfate fractionation (60-90% of saturation) together with DEAE cellulose chromatography occasionally gave a large increase (>100-fold) in specific activity. The results were better when buffers of high ionic strength (0.5 M phosphate) were used for the preparation of the extracts. However, the stability of the enzymatic activity was so slight that the objective of determining the size of the synthetase was not reached.

Following the demonstration that β-alanine is incorporated into a pantetheine moiety bound to protein of high molecular weight in *S. erythreus* CA340 (6), we renewed our study of the biosynthesis of fatty acids in extracts of this organism. First, we looked for some new cofactor that might be required for the enzyme system to function. As shown in Table I, a heat stable factor present in the extract did stimulate the synthesis of fatty acids in a detectable way. The crude enzyme used in this and subsequent work

TABLE I

Stimulation of fatty acid synthetase activity by FMN and a boiled extract supernatant. A mixture containing 10 mμ moles of AcCoA (2.1 Ci/mole), 20 mμ moles of Mal CoA (1.5 Ci/mole), and 100 μmoles of NADPH in 0.1 ml of buffer was prepared. To this was added 0.4 ml of extract or acetone powder homogenate prepared as described in the text. Where indicated, 0.4 ml of boiled supernatant was added and the FMN was at final concentration of 8 micromolar. The final volume was 0.9 ml. The tubes were incubated for 30 minutes at 37°C and the assay was stopped by the addition of 0.9 ml of 50% NaOH. The mixtures were saponified for 2 hours at 100°C and the lipid fraction extracted and counted. The nanomoles of Malonyl CoA incorporated was calculated on the basis of its specific activity.

| | Total dpm Incorporated | Nanomoles of Malonyl CoA incorporated per mg protein |
|---|---|---|
| Extract | 3600 | .31 |
| Acetone powder homogenate + BSA (4 mg) | 3200 | .28 |
| Acetone powder homogenate + boiled supernatant | 7100 | .61 |
| Acetone powder homogenate + FMN | 7500 | .65 |
| Boiled supernatant + BSA (4 mg) | 340 | .03 |

is precipitated from the crude extract with acetone as was suggested by the work of Dimroth (10). Since both the yeast and *Mycobacterium phlei* fatty acid synthetases require FMN for optimal activity (11, 12), we tested this cofactor in our assay system. As seen, FMN when added to the enzyme system stimulated the synthesis of fatty acids to about the same degree as did the supernatant solution of the boiled enzyme extract. The acetone-treated protein was quite stable so long as it was assayed with added FMN. Rapid loss of apparent potency occurred when tubes or columns were exposed to the light. However, when protected the enzyme activity was stable. A slow decay was noted over a 6 week period of storage in a desiccator at 0°C. The enzyme is

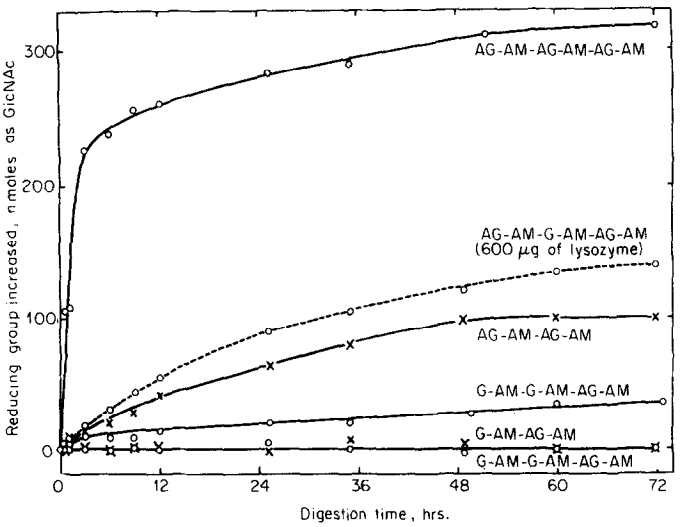


Fig. 2. Hydrolysis of oligosaccharides by lysozyme. The reaction mixture contained 60 μg of lysozyme and each saccharide (GlcN-MurNAc-GlcNAc-MurNAc, 109 nmoles; GlcNAc-MurNAc-GlcNAc-MurNAc, 78 nmoles; GlcNAc-MurNAc-GlcN-MurNAc-GlcNAc-MurNAc, 92 nmoles; GlcN-MurNAc-GlcN-MurNAc-GlcNAc-MurNAc, 135 nmoles; hexasaccharide prepared from B-1-5-1 by N-acetylation, 84 nmoles) in 200 μl of 40 mM ammonium acetate, pH 5.25. After incubation for various time intervals at 37°, aliquots were withdrawn for assaying reducing groups. The increase in reducing groups is expressed in nmoles as N-acetylglucosamine.

sidic linkage between GlcN-MurNAc and the disaccharide unit GlcNAc-MurNAc which formed the reducing end.

In the productive complex with lysozyme, the tetrasaccharide $(GlcNAc\text{-}MurNAc)_2$ is believed to interact with subsite C at the N-acetylglucosamine residue of the nonreducing end (2). The present evidence indicates the requirement for the acetyl group of this N-acetylglucosamine residue in the catalytic cleavage of the glycosidic linkage between the two disaccharide units. The lysozyme resistance of GlcN-MurNAc-GlcNAc-MurNAc moiety in GlcN-MurNAc-GlcN-MurNAc-GlcNAc-MurNAc and GlcNAc-MurNAc-GlcN-MurNAc-GlcNAc-MurNAc are also in accordance with the requirement for the acetyl group of the N-acetylglucosamine residue which interacts with subsite C of the enzyme in the productive complex. Thus,

close relationship of *S. erythreus* with yeasts. Further study of the synthetase will permit more insight into the evolutionary relationships.

*Acknowledgements*

The work described was supported by the National Science Foundation (GB-18157) and the National Institutes of Health (A-109158-04) and the Northwestern University Medical School. Preliminary work done by Dr. F. J. Darby and Mr. Paul T. Borowski is gratefully acknowledged.

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