THE FORMATION OF ENZYME-BOUND ACETOACETATE AND ITS CONVERSION TO LONG CHAIN FATTY ACIDS

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Long chain fatty acid biosynthesis has been shown to proceed through a condensation between malonyl-CoA and acetyl-CoA (Wakil, 1959). The acetyl-CoA ultimately forms the two carbons at the methyl terminal end of the fatty acid; whereas the rest of the carbon chain is formed by multiple two carbon units from malonyl-CoA alone.

The discovery by Lynen in a yeast system that a product of the reaction between malonyl-CoA and acetyl-CoA is enzyme-bound acetoacetate (Lynen, 1960; Lynen, 1961) strongly supports a condensation-decarboxylation mechanism in the elongation of the fatty acid chain by malonyl-CoA. However, proof that this product represented an intermediate in fatty acid synthesis was lacking because of the inability to demonstrate the conversion to fatty acids of the enzyme-bound acetoacetate.

The present paper reports on a soluble enzyme system from E. coli which catalyzes the synthesis of both saturated and unsaturated long chain fatty acids. The system has been resolved into two fractions: a heat-labile fraction (A) precipitating at an ammonium sulfate saturation between 50% and 70% and a heat-stable fraction (Enzyme II) which has been purified 200 fold by acid precipitation from an ammonium sulfate solution at 70% of saturation. In the presence of these two enzyme fractions, both of which are required for the malonyl-CoA-CO_2 exchange reaction (Vagelos, and Alberts, 1960), malonyl-CoA and acetyl-CoA condense to give enzyme-bound acetoacetate. Fractionation of this enzyme-bound acetoacetate indicates binding to Enzyme II. The enzyme-bound intermediate can be isolated and incubated in the presence of Fraction A, malonyl-CoA and TPNH to form long chain fatty acids.

This fatty acid synthesizing system in E. coli is analogous to that previously reported in C. Kluyveri (Goldman, Alberts, and Vagelos, 1961). Each system contains a heat-stable protein, Enzyme II, required both for the malonyl-CoA-CO_2 exchange reaction and for the synthesis of long chain fatty acids. Fraction A in E. coli has the activity of the heat-labile fractions I and III in C. Kluyveri. It is interesting that in spite of these similarities the system in C. Kluyveri synthesizes saturated fatty acids while the system in E. coli synthesizes primarily unsaturated fatty acids.

The requirements for synthesis of the enzyme-bound intermediate are shown in Table I. The fifteen-minute incubation was terminated by the addition of 0.1 ml of 1 N HCl. The precipitate formed was collected by centrifugation and washed three times in 0.2 N acetic acid. The precipitate was dissolved in 0.1 M triethanolamine-HCl buffer at pH 7.5 and an aliquot was counted in an end window gas-flow counter. Similarly a radioactive precipitate can be obtained using radioactive malonyl-CoA and nonradioactive acetyl-CoA showing that malonyl-CoA is incorporated into the precipitated product.

Table I

Requirement for the Synthesis of Enzyme-Bound Intermediate

Incubation conditions	cpm in precipitate
Complete system	22,500
Complete system minus malonyl-CoA	540
Complete system minus Fraction A	360
Complete system minus Enzyme II	130

Complete system contains 70 μmoles of triethanolamine-HCl buffer at pH 7.5, 10 μmoles of 2-mercaptoethanol, 0.05 μmoles of acetyl-1-C^{14}-CoA (200,000 cpm) 0.03 μmoles of malonyl-CoA, 0.4 mg of Enzyme Fraction A and 0.2 mg of Enzyme II in a total volume of 1.0 ml.

To the enzyme-bound radioactive product was added 2 μmoles of carrier acetoacetyl-thioglycollate and the mixture was heated at 80° for two hours in 1 N NaOH. This procedure had previously been shown to release the radioactive product from the protein and to hydrolyze the carrier thiolester. The mixture was acidified and allowed to stand for one hour to decarboxylate the β-keto acid and then the dinitrophenyl hydrazone derivative was made. The radioactive compound formed in this manner chromatographed identically in two solvent systems with the hydrazone derivative of acetone formed from the carrier thiolester. In addition the radioactive compound readily crystallized to constant specific activity with authentic dinitrophenyl hydrazone of acetone. On this basis the enzyme-bound radioactive compound was considered to be acetoacetate.

To identify the protein to which the acetoacetate is bound, 15 mg. of Fraction A were added to the washed precipitate formed as in Table I. An ammonium sulfate fractionation of this mixture showed that most of the protein precipitated between 40% and 60% saturation and nearly all of it had precipitated by 80% saturation. Yet 70% of the enzyme-bound radioactivity remained in the supernatant solution and was recovered by acid precipitation. This fractionation of the radioactivity thus almost exactly parallels the separation of Enzyme II from fraction A by ammonium sulfate. The radioactive compound obtained by acid precipitation and resolved of Fraction A was chromatographed on DEAE-cellulose with carrier Enzyme II. In the effluent fractions the radioactivity recovered exactly paralleled Enzyme II activity as assayed in the Malonyl-CoA-CO_2 exchange reaction in the presence of an excess of fraction A (Vagelos, and Alberts, 1960).

The requirements for the conversion of enzyme-bound acetoacetate to long chain fatty acids are shown in Table II. Long chain fatty acids were extracted and assayed as previously described (Goldman et al., 1961) and their identity confirmed by gas-liquid chromatography.

The radioactive fatty acids formed in the second incubation could be the result of the direct incorporation of the radioactive acetoacetate into

Table II

Requirement for Conversion of Intermediate to Long Chain Fatty Acids

Incubation conditions	cpm in long chain fatty acids
Complete system	102
Complete system minus malonyl-CoA	0
Complete system minus TPNH generating system	0
Complete system minus Enzyme Fraction A	0

Complete system contains 50 μmoles of triethanolamine-HCl buffer at pH 7.5, 10 μmoles of 2 mercaptoethanol, 0.03 μmoles of malonyl-CoA, 1.0 mg of Enzyme Fraction A, 200 cpm of enzyme-bound intermediate and a TPNH generating system (0.02 μmoles of TPN, 3 μmoles of glucose 6-phosphate and 0.13 K units of glucose 6-phosphate dehydrogenase) in a total volume of 1 ml. Incubation is for 30 minutes with air as the gas phase.

the first four carbons at the methyl end of the fatty acids or it could result from the reversal of acetoacetate formation with the production of acetyl-CoA or malonyl-CoA which in the presence of malonyl CoA and TPNH would be incorporated into fatty acids. To examine the possibility that the enzyme-bound intermediate acted as a source of malonyl-CoA, the enzyme-bound product formed from malonyl-1,3-C^{14}-CoA and acetyl-CoA was isolated and incubated with TPNH and unlabeled malonyl-CoA in a manner similar to that of Table II except on a larger scale. 2520 cpm were obtained in long chain fatty acids. These fatty acids were isolated as their methyl esters by gas liquid chromatography and 1300 cpm were recovered in methyl esters of octadecenoic acids (oleic or vaccenic). A permanganate periodate oxidation (von Rudloff, 1956) of these mono-olefinic acids produced monocarboxylic acids from the carbon chain on the methyl terminal side of the double bond and dicarboxylic acids from the

carbon chain on the carboxyl terminal side of the double bond. Essentially all of the radioactivity was accounted for in the monocarboxylic acids. If a significant quantity of radioactive malonyl-CoA had formed during the second incubation and had been responsible for the radioactivity in these unsaturated fatty acids one would have expected a considerable fraction of the radioactivity from the intermediate to appear in the dicarboxylic acids. The fact that the monocarboxylic acids retain all of the label of the starting 18 carbon acids is consistent with the radioactive, enzyme-bound acetoacetate being incorporated directly into the long chain fatty acids rather than acting as a source of malonyl-CoA.

The possibility remained that the enzyme-bound acetoacetate acted as a source of radioactive acetyl-CoA which in the second incubation was incorporated into the methyl end of the fatty acids synthesized. This possibility was eliminated, however, by showing that the addition of a pool of nonradioactive acetyl-CoA (0.03 μmoles) to the second incubation failed to dilute the radioactivity incorporated into fatty acids under conditions of Table II.

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