Enzyme Specificity as a Factor in Regulation of Fatty Acid Chain Length in Escherichia coli

Abstract. *Various acyl-acyl carrier protein intermediates in saturated and unsaturated fatty acid biosynthesis were tested as substrates for β-ketoacyl-acyl carrier protein synthetase. With both classes of substrates the condensing enzyme in fatty biosynthesis demonstrates specificities which indicate that it might be an important factor in determining fatty acid chain length in* Escherichia coli.

The elongation of the fatty acyl chain during fatty acid biosynthesis is catalyzed by the enzyme β-ketoacyl-acyl carrier protein (ACP) synthetase by the following reaction.

Fatty acyl-ACP + malonyl-ACP ⇌
β-ketoacyl-ACP + ACP + CO_2

This condensing enzyme from *Escherichia coli* has been purified to homogeneity (*1*). With acetyl-ACP as substrate, the enzymatic reaction involves the initial transfer of the acetyl group from acetyl-ACP to the enzyme, forming an acetyl-enzyme intermediate. The acetyl group is then transferred from the enzyme to malonyl-ACP forming acetoacetyl-ACP with the release of CO_2.

The availability of the pure condensing enzyme and some newly synthesized substrates permitted the further investigation of two interesting biological problems. (i) Does this enzyme catalyze all the condensations in the biosynthesis of both saturated and unsaturated fatty acids? (ii) Can the specificity of this enzyme explain the accumulation of fatty acids of particular chain lengths in the cell? Some preliminary data in these studies have been presented (*2*).

β-Ketoacyl-ACP synthetase from *E. coli* B was purified and assayed by the methods of Greenspan *et al.* (*1*). *cis*-3-Decenoic acid was prepared according to Hofmann *et al.* (*3*) with the hydrogenation apparatus described by Brown and Brown (*4*). The product was 96 percent homogeneous, as judged by gas-liquid chromatography [10 percent diethyleneglycol succinate (DEGS) on Chromosorb-W DMGS-AW, Varian Aerograph]. The major product was further characterized by chromic acid oxidation (*5*). Heptanoic acid was identified as the major cleavage product by chromatography of the methyl ester with an authentic standard on two gas-liquid systems (10 percent DEGS and 3 percent SE30).

The synthesis of *cis*-5-dodecenoic acid was achieved by the following sequence of reactions. Heptyltriphenylphosphonium iodide was synthesized by the reaction of heptyl iodide and triphenylphosphine (at 80°C for 20 minutes) in the absence of solvent. *cis*-5-Dodecenol was prepared by a Wittig reaction (*6*) between 5-hydroxypentanal and the alkylidenetriphenylphosphorane obtained by the addition of heptyltriphenylphosphonium iodide to a mixture of sodium ethoxide and dimethyl formamide. A high yield of the required *cis*-isomer was obtained by the use of the modified procedure for the Wittig reaction developed by Bergelson and Shemyakin (*7*). After purification the alcohol was oxidized with Jones reagent (*8*) to give the required product, *cis*-5-dodecenoic acid. The product was identified by gas-liquid chromatography and nuclear magnetic resonance. These methods also indicated that the product was homogeneous, except for a small quantity of the *trans*-isomer (less than 5 percent). All acyl-ACP substrates used were prepared as previously described (*9*).

β-Ketoacyl-ACP synthetase was assayed with various fatty acyl-ACP intermediates of the saturated and unsaturated fatty acid biosynthetic pathways as substrates (Table 1). Reactions with acetyl-ACP, decanoyl-ACP, and dodecanoyl-ACP gave approximately similar results for both the Michaelis constant (K_m) and the maximum velocity (V_{max}). The enzyme was slightly less active with tetradecanoyl-ACP. However, it was completely inactive with hexadecanoyl-ACP even when this substrate was tested with 50 times more enzyme than that used with the acyl-ACP's of shorter chain length. Assay of the enzyme with *cis*-3-decenoyl-ACP and *cis*-5-dodecenoyl-ACP, two early intermediates in the synthesis of unsaturated fatty acids, indicated that they were both as active as acetyl-ACP or decanoyl-ACP and that they have similar K_m's. Activity of the *cis*-9-hexadecenoyl-ACP was decreased to approximately one-fifth of the rate of the *cis*-3-decenoyl-ACP or *cis*-5-dodecenoyl-ACP. The *cis*-11-octadecenoyl-ACP was completely inactive, although tested with 50 times the enzyme concentration used above.

To determine whether the palmityl-ACP and *cis*-vaccenyl-ACP used were physiologically competent preparations, they were tested individually as acyl donors to glycerol-3-phosphate in the presence of a particulate acyltransferase of *E. coli* (*10*). An efficient acyl transfer reaction with each substrate occurred, and the expected lysophosphatidic acids were formed.

The product of the synthetase condensation of *cis*-3-decenoyl-ACP with [2-^{14}C]malonyl-ACP, cis-5-β-ketododecenoyl-ACP, was identified after reduction by reduced nicotinamide adenine dinucleotide phosphate in the presence of β-ketoacyl-ACP reductase (*11*). The free *cis*-5-β-hydroxydodecenoic acid was obtained by Pronase hydrolysis (*12*) and was cleaved in a periodate-permanganate-oxidation (*13*). The ^{14}C-labeled dicarboxylic acid product chromatographed, as the methyl ester, with authentic β-hydroxyglutaric dimethyl ester as judged by gas-liquid chromatography on 1 percent SE30 and on 10 percent DEGS columns. The product of the condensation of decanoyl-ACP with [2-^{14}C]malonyl-ACP, β-ketododecanoyl-ACP, was identified, after enzymatic reduction of the β-keto group, as the methyl ester of β-hydroxydodecanoic acid. In thin-

Table 1. Activity of β-ketoacyl-ACP synthetase with various intermediates of fatty acid synthesis. All assays were carried out according to the method of Greenspan *et al.* (*1*). Kinetic constants were determined from Lineweaver-Burk plots of the data obtained. The maximum velocity (V_{max}) is expressed as the number of micromoles of product formed per minute per milligram of enzyme; N.A., no activity.

Intermediate	Maximum enzyme concentration (μg/ml)	K_m (× $10^{-5}M$)	V_{max}
Acetyl-ACP	4.0	5.2	2.8
Decanoyl-ACP	4.0	3.3	2.8
Dodecanoyl-ACP	4.0	2.7	0.97
Tetradecanoyl-ACP	4.0	2.8	0.31
Hexadecanoyl-ACP	200.0		N.A.
cis-3-Decenoyl-ACP	4.0	7.1	1.9
cis-5-Dodecenoyl-ACP	3.4	2.0	1.7
cis-9-Hexadecenoyl-ACP	34.0	3.7	0.37
cis-11-Octadecenoyl-ACP	200.0		N.A.

layer chromatography on silver-impregnated silica gel (*14*) with a developing system of chloroform and 0.75 percent ethanol, the enzymatic product chromatographed with the authentic compound. In addition, mass spectroscopy of the trimethylsilyl derivative of the methyl ester was used to identify the β-hydroxydodecanoic acid.

Our results, along with those of Toomey and Wakil concerning the shorter chain saturated acyl-ACP's (*15*), indicate that this condensing enzyme can account for both saturated and unsaturated products of fatty acid biosynthesis in *E. coli*. The specificities indicated by the kinetic data are consistent with the reports of Lennarz *et al.* (*16*) and of Knivett and Cullen (*17*), who found that predominating among saturated fatty acids in whole cells is palmitate and among unsaturated fatty acids are palmitoleate and *cis*-vaccenate, or their cyclopropane derivatives. Chain termination in the yeast fatty acid synthetase is also strongly dependent on the efficiency of the rate-limiting reaction catalyzed by the condensing enzyme of that system (*18*).

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References and Notes

1. M. D. Greenspan, A. W. Alberts, P. R. Vagelos, *J. Biol. Chem.* **244**, 6477 (1969).
2. M. D. Greenspan, C. H. Birge, P. R. Vagelos, *Fed. Proc.* **28**, 537 (1969).
3. K. Hofmann, W. M. O'Leary, C. W. Yoho, T. Lin, *J. Biol. Chem.* **234**, 1672 (1959).
4. C. A. Brown and H. C. Brown, *J. Amer. Chem. Soc.* **84**, 2829 (1962).
5. F. Davidoff and E. D. Korn, *J. Biol. Chem.* **238**, 3199 (1963).
6. G. Wittig and U. Schoellkopf, *Org. Syn.* **40**, 66 (1960).
7. I. D. Bergelson and M. M. Shemyakin, *Angew. Chem. Int. Ed. Engl.* **3**, 250 (1964).
8. A. Bouers, C. Djerassi, R. R. Engle, *J. Org. Chem.* **21**, 1547 (1956).
9. C. H. Birge, D. F. Silbert, P. R. Vagelos, *Biochem. Biophys. Res. Commun.* **29**, 808 (1967).
10. H. van den Bosch and P. R. Vagelos, *Biochim. Biophys. Acta*, in press.
11. P. R. Vagelos, A. W. Alberts, P. W. Majerus, *Meth. Enzymol.* **14**, 60 (1969).
12. L. R. Kass, D. J. H. Brock, K. Bloch, *J. Biol. Chem.* **242**, 4418 (1967).
13. G. Scheuerbrandt and K. Bloch, *ibid.* **237**, 2064 (1962).
14. C. B. Barrett, M. S. S. Dallas, R. B. Padley, *J. Amer. Oil Chem. Soc.* **40**, 580 (1963).
15. R. E. Toomey and S. J. Wakil, *J. Biol. Chem.* **241**, 1159 (1966).
16. W. J. Lennarz, R. J. Light, K. Bloch, *Proc. Nat. Acad. Sci. U.S.* **48**, 840 (1962).
17. V. A. Knivett and J. Cullen, *Biochem. J.* **103**, 299 (1967).
18. M. Sumper, D. Oesterhelt, C. Riepertinger, F. Lynen, *Eur. J. Biochem.* **10**, 377 (1969).
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Electron Probe X-ray Microanalysis of a Normal Centriole

Abstract. *A scanning spectrometer with lithium fluoride, ammonium dihydrogen phosphate, and gypsum crystals was used to detect the x-ray spectrum of a normal centriole, the transmitted electron image of which was used to focus the exciting electron beam to the size, shape, and position of the centriole in a 700-angstrom section of intact tissue.*

We have used electron microprobe image-forming and x-ray microanalysis in the identification of the elements present in the centriole. Although huge by molecular standards, centrioles are so small as to be barely distinguishable at the limit of visible light microscopy, and then in only the thinnest of sections.

Their study requires high-resolution magnification ranging from ×2000 to ×10,000 of sections not much in excess of 1000 Å. Essentially nothing is known of the molecular species which massively aggregate into the nine triplet microtubules of the centriolar wall. Conventional analytical methods have not been applied to centrioles for the following reasons: (i) these organelles measure only 0.2 by 0.5 μm, (ii) they occur generally in the number of only two per cell, and (iii) no one has managed to isolate them from other cell constituents. Even from electron microscopy of random sections of intact tissue one cannot consistently determine whether one is observing an inside or an outside member of the pair or whether one is approaching or retreating from the intercentriolar angle. However, a procedure has been perfected which consistently yields information from consecutive sections of intact tissues prepared for ultramicrotomy and electron microscopy by conventional methods (*1*).

This procedure was used in the location and identification of the centrioles of the distal convoluted tubule of a normal guinea pig kidney. The sections were then analyzed in an electron microscope-microanalyzer (EMMA-4, AEI) (*2*). In preliminary nondispersive analyses and spectrometer scans we found that the sections contained very small amounts of the various elements. To obtain sufficient x-ray output, we used a 100-kv accelerating potential and maximum available beam current. Discrimination at the resultant level of output precluded nondispersive detection. Thus we had to use crystal spectrometer scans to locate all of the heavier elements which were present. To reduce the chance of specimen damage due to the high beam current, we used relatively fast scanning rates (crystal movements of 0.25 to 0.5 inch/min).

Figure 1 is a survey electron photomicrograph of the section selected for detailed analysis. The 12 cells comprising the intact tubular wall are overlapped in such a way that the section passes through various amounts of cytoplasm and through only three nuclei. All of the cytoplasmic organelles ranging in size up to 1.0 μm are mitochondria, with one exception. The outside centriole, in nearly perfect cross section, whose analysis is here reported, is distinguishable from the other organelles by its intense electron opacity, its circular form, and the constant curl-div configuration (*3*) of its microtubules.

The EMMA-4 electron beam was focused to precisely the size and shape of this particular centriole, simultaneously with its exact registration onto that target. The spectrometer was then scanned throughout its range of wavelengths, lithium fluoride, ammonium dihydrogen phosphate, and gypsum crystals being used in turn. The three traces obtained showed the presence of those elements listed in Table 1.

The ammonium dihydrogen phosphate crystal scanned at a rate of 0.5 inch/min with a chart speed of 1.0 inch/min yielding a horizontal calibration of 0.443 Å/inch (Fig. 2). Full-scale vertical deflection was made equal to a spectrometer output of 50 count/sec. Each of the elemental peaks encountered in these scans was confirmed by subsequent detailed examination. Without a change in its size, the microbeam was then moved to positions immediately adjacent to but not including