SYNTHESIS OF ACETOACETYL-CoA BY BOVINE MAMMARY FATTY ACID SYNTHASE

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1. Introduction

Fatty acid synthase (FAS) in animal tissues and yeast is a polyfunctional enzyme complex which catalyzes the synthesis of saturated fatty acids by the chain elongation of acetyl-CoA, the primer, by successive additions of two-carbon units derived from malonyl-CoA in the presence of NADPH. The 6 different catalytic activities required for each chain elongation step are acetyl transacylase, malonyl transacylase, a decarboxylation–condensation reaction, β-ketacyl reductase, β-hydroxyacyl dehydratase and enoyl reductase. Recent investigations have shown some striking differences in the catalytic activities of FAS from the mammalian sources on the one hand and avian tissues and yeast on the other [1,2]. Among these is the occurrence of the transacylation of the acetoacetyl group between FAS and CoA [2]. This suggested that mammalian enzyme might have the ability to synthesize acetoacetyl-CoA from acetyl-CoA and malonyl-CoA, provided NADPH is absent. Triacetic acid lactone (TAL) is the main product of such a reaction when pigeon liver or yeast is the enzyme source [3,4]. Bovine mammary FAS was indeed found to produce acetoacetyl-CoA in addition to TAL. However, if the acetoacetyl-CoA produced is continuously reduced using NADH and 3-hydroxy acyl-CoA dehydrogenase TAL is not produced. 3-Hydroxybutyryl-CoA is then the product. The coupling of the production of acetoacetyl-CoA to its reduction formed the basis for the development of a convenient assay for the decarboxylation–condensation reaction of FAS.

2. Materials and methods

Acetyl-CoA, malonyl-CoA and [2-^{14}C]malonyl-CoA, were obtained from commercial sources and assayed as in [5]. Triacetic acid lactone (TAL) was prepared by the method in [6]. Its identity was established from its melting point, molar absorbancy, chromatographic behavior [4] and from its IR and NMR spectra. Pig heart 3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35) was obtained from Sigma.

FAS was purified from lactating bovine mammary gland and assayed as in [5].

For the identification of the CoA-bound acids produced in the different reactions, the incubation was terminated by immersion of the tube containing the reaction mixture in water at 60°C for 2 min. After the addition of appropriate carrier compounds 5 μmol $NH_2OH \cdot HCl$ (pH 7.0) was added and the mixture allowed to stand for 30 min at room temperature. The CoA esters of acids other than acetoacetate are quantitatively converted to their corresponding hydroxamates, while acetoacetyl-CoA is converted to methylisoxazolone [7]. These derivatives were extracted, desalted and chromatographed on filter paper as in [7].

3. Results and discussion

The formation of acetoacetyl-CoA by FAS from acetyl-CoA and malonyl-CoA was ascertained in preliminary studies spectrophotometrically from the oxidation of NADH added to the incubation mixture along with 3-hydroxyacyl-CoA dehydrogenase. TAL does not oxidize NADH in the presence or absence of 3-hydroxyacyl-CoA dehydrogenase. The formation of acetoacetyl-CoA by the fatty acid synthetase was established by two additional procedures:

(1) Acetoacetyl-CoA formed was reacted with neutral hydroxylamine and methyl isoxazolone, the reaction product [7] was identified chromatographically [8]. The results are shown in fig.1.

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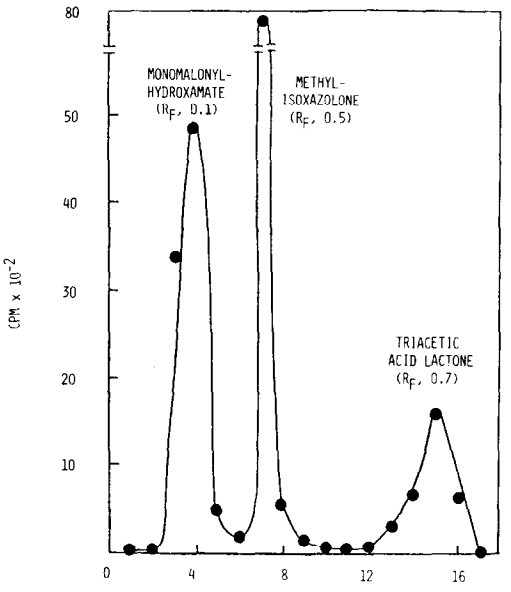


Fig.1. Formation of methyl isoxazolone, the reaction product of acetoacetyl-CoA and neutral hydroxylamine [7]. The incubation mixture contained in 0.5 ml, 0.1 M potassium phosphate buffer, 1.0 nM dithiothreitol, 250 μM acetyl-CoA, 500 μM [2-^{14}C]malonyl-CoA (spec. act. 1.1 Ci/mol) and 25 μg FAS. The mixture without malonyl-CoA was preincubated for 8 min at 37°C and the reaction was initiated by the addition of malonyl-CoA. After a 10 min incubation the reaction was terminated by immersing the container in water kept at 60°C for 2 min. Then, 0.25 μmol each of carrier acetoacetyl-CoA, TAL and 5 μmol neutral hydroxylamine were added. The reaction products were extracted and chromatographed on paper as in section 2. The filter paper was dried, cut into 1 cm wide pieces and counted for ^{14}C activity in a liquid scintillation spectrometer.

Although the nature of the reaction product of triacetic acid lactone with hydroxylamine was not established, the identity of the peak of R_F 0.7 as TAL or a product derived from TAL was established in control experiments. The formation of acetoacetyl-CoA as the major product is obvious from the radioactivity associated with methyl isoxazolone.

(2) Acetoacetyl-CoA formed was reduced continuously using 3-hydroxyacyl-CoA dehydrogenase and NADH. 3-Hydroxybutyryl-CoA formed was converted to 3-hydroxybutyryl hydroxamate by the addition of neutral hydroxylamine and the hydroxamate identified chromatographically [8]. The results are shown in fig.2. TAL was not produced under these conditions.

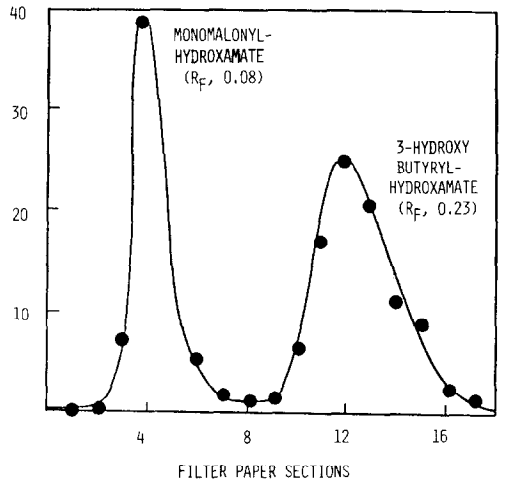
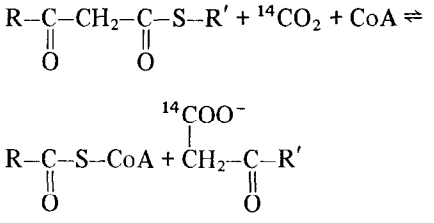


Fig.2. Evidence for the formation of acetoacetyl-CoA by its reduction to 3-hydroxybutyryl-CoA and its conversion to hydroxamate. The incubation mixture contained in 0.5 ml the ingredients mentioned under fig.1 and in addition 125 μM NADH and 100 μg 3-hydroxyacyl-CoA dehydrogenase. The reaction was initiated and terminated as described under fig.1. Carrier 3-hydroxybutyryl-CoA and TAL were added followed by the addition of neutral hydroxylamine. The reaction products were extracted and chromatographed.

It was obvious from the above experiments that the coupling of the formation of acetoacetyl-CoA to its reduction using NADH and 3-hydroxyacyl-CoA dehydrogenase would constitute a convenient spectrophotometric assay for the decarboxylation–condensation reaction of fatty acid synthase. This partial reaction of the enzyme complex from yeast and animal tissues is currently assayed by measuring the fixation of $^{14}CO_2$ into malonylthiol ester according to the reaction:



where R is either methyl [9] or pentanyl group [10] and R′ is either CoA [10] or *N*-acetylcysteamine [9]. Since this reaction is in the reverse direction to that which normally occurs during the fatty acid synthesis, and, besides, is energetically highly unfavorable,

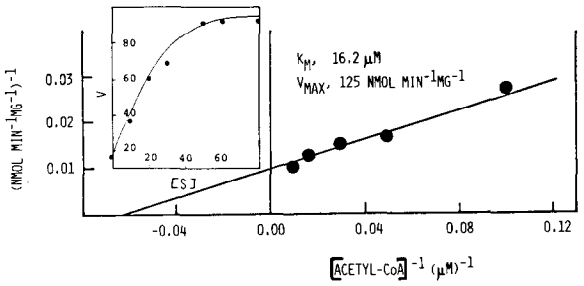


Fig.3. Effect of acetyl-CoA concentration on the rate of the condensing reaction in the coupled spectrophotometric assay. The reaction mixture in a final volume of 0.4 ml contained: 100 mM phosphate buffer (pH 7.0), 1 mM dithiothreitol, 25 μg FAS (spec. act. 650), 60 μg 3-hydroxyacyl-CoA dehydrogenase, varying amounts of acetyl-CoA, 125 μM NADH and 50 μM malonyl-CoA. The mixture without NADH and malonyl-CoA was preincubated at 37°C for 8 min, then NADH added, followed 2 min later by the addition of malonyl-CoA. The oxidation of NADH was followed spectrophotometrically from the decrease in absorbance at 340 nm. The activity at zero concentration of acetyl-CoA is most likely due to the production of acetyl-S-Enz by the decarboxylation of malonyl-CoA [17].

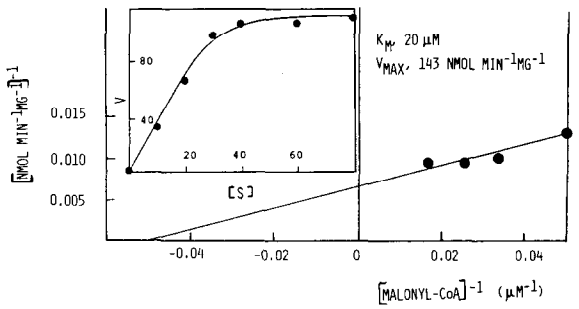


Fig.4. Effect of malonyl-CoA concentration on the condensing reaction. Incubation conditions were as in fig.3 except for the fixed concentration of acetyl-CoA, at 50 μM and varying concentration of malonyl-CoA.

relatively high concentrations of the various substrates and FAS are required to obtain measurable rates. Even though the condensing reaction has received much attention in investigations on the mechanism of fatty acid synthesis [11–14], and is the only partial reaction of the enzyme complex requiring the dimeric form of the enzyme [15], this reaction has not been characterized, undoubtedly because of the inadequacy of the assay procedure.

Using the spectrophotometric assay of the oxidation of NADH, involving the coupling of the production of acetoacetyl-CoA with its reduction catalyzed by 3-hydroxyacyl-CoA dehydrogenase, relatively high rates were obtained for the condensing reaction. As the equilibrium of the dehydrogenase catalyzed reaction lies far towards the reduction product, at pH 7.0, the rate of oxidation of NADH truly represents the condensing reaction provided FAS is limiting and the dehydrogenase is in excess. The effect of acetyl-CoA and malonyl-CoA concentrations of the condensing reaction, under the proper conditions, are shown in fig.3,4. The rates of ⩾100 nmol acetoacetyl-CoA formed . min^{-1} . mg enzyme^{-1} is in sharp contrast to those of 0.2 to 3.5 reported for yeast, chicken liver, pigeon liver and rat mammary FAS using the assay involving CO_2 fixation [9,11,15,16].

The production of acetoacetyl-CoA by FAS and its utilization for fatty acid synthesis suggest a role for FAS in ketone body metabolism. This role remains to be clarified.

Acknowledgements

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References

[1] Abdinejad, A. and Kumar, S. (1981) Arch. Biochem. Biophys. 208, 135–145.
[2] Dodds, P. F., Guzman, M. G. F., Chalberg, S. C., Anderson, G. J. and Kumar, S. (1981) J. Biol. Chem. 256, 6282–6290.
[3] Nixon, J. E., Putz, G. R. and Porter, J. W. (1968) J. Biol. Chem. 243, 5471–5478.
[4] Yalpani, M., Willecke, K. and Lynen, F. (1969) Eur. J. Biochem. 8, 495–502.
[5] Strom, K., Galeos, W. L., Davidson, L. A. and Kumar, S. (1979) J. Biol. Chem. 254, 8153–8158.
[6] Collie, J. N. (1981) J. Chem. Soc. 59, 607–617.
[7] Avena, R. M. and Kumar, S. (1962) Anal. Biochem. 4, 514–516.
[8] Kumar, S. and Avena, R. M. (1963) Anal. Biochem. 5, 265–267.
[9] Lynen, F. (1969) Methods Enzymol. 14, 17–33.
[10] Muesing, R. A. and Porter, J. W. (1975) Methods Enzymol. 35, 45–59.
[11] Stoops, J. K. and Wakil, S. J. (1981) J. Biol. Chem. 256, 5128–5133.

[12] Kumar, S., Opas, E. and Alli, P. (1980) Biochem. Biophys. Res. Commun. 95, 1642–1649.
[13] Andrews, D. E., Musik, J. L. and Stoops, J. K. (1981) Fed. Proc. FASEB 40, 1780 (abst. 1383).
[14] Ahmad, P. M., Chisner, M. B., Ahmad, F., Clements, P. R. and Burden, R. E. (1981) Fed. Proc. FASEB 40, 1794 (abst. 1463).
[15] Kumar, S., Dorsey, J. A., Muesing, R. A. and Porter, J. W. (1970) J. Biol. Chem. 245, 4732–4744.
[16] Smith, S., Agradi, E., Libertini, L. and Dileepan, K. N. (1976) Proc. Natl. Acad. Sci. USA 73, 1184–1188.
[17] Katiyar, S. S., Briedis, A. V. and Porter, J. W. (1974) Arch. Biochem. Biophys. 162, 412–420.