Regulation of de novo Fatty Acid Biosynthesis in the n-Alkane-utilizing Yeast, Candida 107

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(Received 25 April 1973)

SUMMARY

Growth of Candida 107 on n-alkanes (C_{10}, C_{16} or a mixture) completely repressed formation of acetyl-CoA carboxylase and partially repressed the fatty acid synthetase complex. As all fatty acids must then be derived directly from the substrate no matter what its chain length, the yeast must be able to elongate even-chain acids (C_{16} to C_{14}) and modify, by unknown reactions, odd-chain acids to give even-chain acids. Short-term control of fatty acid biosynthesis appears to be by long-chain (C_{16} or C_{18}) fatty acyl-CoA esters feedback-inhibiting the activities of both acetyl-CoA carboxylase and fatty acid synthetase. n-Alkanes, n-alcohol, free fatty acids or C_{12} and C_{14} acyl-CoA esters, had little or no effects on these enzymes. Extracts from n-alkane-grown yeast inhibited the carboxylase in extracts from glucose-grown yeast, the pattern of inhibition being similar to that observed with hexadecyl-CoA.

INTRODUCTION

De novo fatty acid biosynthesis in yeast is catalysed by two soluble enzyme systems: acetyl-CoA carboxylase (EC. 6.4.1.2), equation 1, and the multienzyme complex known as the fatty acid synthetase (Sumper & Lynen, 1972), equation 2

\[
\text{Acetyl-CoA} + \text{HCO}_3^- + \text{ATP} \rightarrow \text{malonyl-CoA} + \text{ADP} + \text{P}_i
\]  

\[
\text{Acetyl-CoA} + 7 \text{Malonyl-CoA} + 14 \text{NADPH} + 14\text{H}^+ \rightarrow \text{hexadecyl-CoA} + 7\text{CoA} + 7\text{CO}_2 + 14\text{NADP} + 6\text{H}_2\text{O}
\]  

Acetyl-CoA carboxylase is subject to allosteric regulation; it is activated by citrate and Mg^{2+} and inhibited by long-chain acyl-CoA thioesters in liver or adipose tissues (Vagelos, 1971; Guynn, Veloso & Veech, 1972) and in crude extracts of Saccharomyces cerevisiae (White & Klein, 1965, 1966; Rasmussen & Klein, 1968), though Matsushashi (1969) has reported that the yeast enzyme is not so affected. This enzyme is repressed in Lactobacillus plantarum when grown in the presence of unsaturated fatty acids (Henderson & McNeil, 1966, 1967; Birnbaum, 1970; Weeks & Wakil, 1970) and partially repressed in starved rats (Majerus & Kilburn, 1969) when lipolysis is presumably stimulated, but there have been no reports of its repression in yeasts (Hunter & Rose, 1971).

The fatty acid synthetase complex in animals is stimulated by certain hexose phosphates (Wakil, Goldman, Williamson & Toomey, 1966) and is inhibited by hexadecyl-CoA (Tubbs & Garland, 1964; Dorsey & Porter, 1968) which also inhibits the yeast enzyme (Lust & Lynen, 1968). Partial repression of the enzyme complex occurs in starved animals (Butterworth et al. 1966; Dahlen, Kennan & Porter, 1968; Burton, Collins, Kennan & Porter, 1969). Changes in the level of the related enzyme system have been reported in Lactobacillus plantarum (Birnbaum, 1970; Weeks & Wakil, 1970) but not in yeasts.
Addition of fatty acids, as such, to the medium can inhibit yeast growth (Bell, 1971, 1973; Gill & Ratledge, 1972) although small amounts can be used by *Saccharomyces cerevisiae* (Suomalainen & Kerän, 1968). A more suitable system for the study of control of *de novo* synthesis of fatty acids could therefore be to use alkane-utilizing micro-organisms. In these organisms, fatty acids are directly derived from alkanes (Davis, 1964; Mizuno et al. 1966; see also Ratledge, 1970) without inhibition of growth. These fatty acids, in yeasts, may then be metabolized in one or more of three ways depending upon their chain length: (i) from C_{13} to C_{18} direct incorporation into lipids can occur; (ii) from C_{9} to C_{15} (and maybe higher) elongation of the chain is indicated to occur; and (iii) degradation to acetate occurs to some extent with all alkanes (Trust & Millis, 1970; Chenouda & Jwanny, 1972; Hornei, Köhler & Weide, 1972; Thorpe & Ratledge, 1972; Hug & Fiechter, 1973). In *Candida* 107, which can oxidize *n*-alkanes to fatty acids even when growing on glucose (Ratledge, 1968; Gill & Ratledge, 1973a), one might therefore expect the system for *de novo* fatty acid synthesis to be regulated by repression of enzyme synthesis or feedback inhibition or both. We have now investigated such regulation; a preliminary communication of part of this work has already been made (Gill & Ratledge, 1973b).

**METHODS**

*Growth of the organism. Candida* 107 was grown in shake-culture on a glucose+yeast extract+salts medium (Gill & Ratledge, 1972); after 18 h all glucose had been consumed. To starve the yeast, incubation was continued for a further 24 h. To adapt the yeast to complete alkane utilization, 20 ml of the appropriate sterile alkane was added to each litre of culture after glucose exhaustion and then incubation continued for a further 24 h. Alternatively, the yeast was grown in continuous culture at a dilution rate of 0·1 h^{-1} on 5% *(v/v)* n-alkanes (C_{13} to C_{16} fraction) in a 10-l fermenter (3-l working vol.). The basal salts and vitamins of the medium were as described by Ratledge (1968) except that (NH_{4})_{2}SO_{4} was at 2·5 g/l and Span 81 was added to 0·1% *(v/v)*.

*Preparation of cell-free extracts and assay of enzymes. Candida* 107 was harvested by centrifuging for 5 min at 6000 g, washed twice with 0·1 M-phosphate buffer, pH 7·0, and then, as a slurry in fresh buffer, disrupted by passage through a French press. The material was centrifuged for 15 min at 10000 g to remove whole yeast and debris and the supernatant solution re-centrifuged at 15000 g for 30 min to remove mitochondria. The final supernatant was filtered with suction through Whatman fibre-glass paper (grade A) to remove solidified fat. The protein concentration, determined by the method of Lowry, Rosebrough, Farr & Randall (1951), was adjusted for all cell-free extracts to 10 mg/ml with 0·1 M-phosphate buffer. As the effect of some inhibitors, principally long-chain acyl-CoA thioesters, can be reversed by their binding to protein (Numa, Bortz & Lynen, 1965; Marquis, Francesconi & Vilee, 1968), a constant protein concentration was maintained to validate comparisons of inhibitory effects between different enzyme preparations.

Acetyl-CoA carboxylase (acetyl-CoA:carbon dioxide ligase (ADP) EC. 6.4.1.2) was assayed using a method based on that described by Matsuhashi (1969) but omitting EDTA and including 2 mM-Mn^{2+}. The final volume was 1·0 ml, the reaction being started by adding 0·1 ml of cell-free extract. The enzyme preparation and the assay mixture were held at 30 °C for 10 min before mixing. [2-^{14}C]Acetic acid (2 μmol; 1 μCi) with CoA were sometimes replaced in the assay by [2-^{14}C]acetyl-CoA (1 μmol; 1 μCi). Effectors of the enzyme were added to the assay mixture before adding the enzyme. The reaction was stopped after 15 min at 30 °C by adding 0·5 ml 5 M-KOH, containing 1 μmol hexadecanoic acid to act as carrier. Hexadecanoic acid was recovered by solvent extraction and, after appropriate washing, its
radioactivity was counted by liquid scintillation. The activity of the enzyme in all preparations used was linear with respect to time of incubation for up to 20 min, and with respect to protein concentration was linear up to 1.5 mg protein/ml of assay mixture in the presence of 75 mM-citrate.

Fatty acid synthetase was assayed according to Lynen (1969), except that bovine serum albumin was omitted. Fatty acids formed from [2-14C]malonyl-CoA were assayed by the same method used in determination of acetyl-CoA carboxylase activity.

Preparation of CoA-esters. [2-14C]Acetyl-CoA was prepared from the labelled anhydride according to Simon & Shemin (1953); [2-14C]malonyl-CoA was prepared from the free acid according to Trams & Brady (1960) and long-chain acyl-CoA esters were prepared from the corresponding acyl chlorides by the method of Seubert (1960).

Chemicals used. All materials used in enzyme assays were obtained from Sigma Chemical Co. Ltd, London. Radiochemicals were obtained from the Radiochemical Centre, Amersham, Buckinghamshire. n-Decane, n-hexadecane and n-decanol, each at 99% purity, were from Koch-Light Laboratories Ltd, Colnbrook, Buckinghamshire. The fraction of n-alkanes used for cultivation of the yeast contained 2.3% C_{13}, 55.7% C_{14}, 35.3% C_{15} and 6.6% C_{16} n-alkanes and was a gift from Texaco Ltd, London.

RESULTS

Glucose-grown Candida 107

Activation and inhibition of fatty acid synthetase complex. The fatty acid synthetase complex, as isolated from pigeon liver, is activated maximally by fructose 1,6-diphosphate but only poorly by inorganic phosphate (Wakil et al. 1966). The enzyme from Candida 107, however, showed only poor stimulation of activity by fructose 1,6-diphosphate alone and appeared to have an absolute requirement for inorganic phosphate for maximum stimulation (Fig. 1). Subsequent assays of the fatty acid synthetase complex were therefore always performed in 100 mM-phosphate buffer, without fructose 1,6-diphosphate. Fructose 1,6-diphosphate may have exerted its slight activation in tris buffer through hydrolysis by an appropriate phosphatase, to yield some inorganic phosphate in these assays.

In Saccharomyces cerevisiae, the fatty acid synthetase complex is inhibited by long-chain acyl-CoA esters (Lust & Lynen, 1968). In Candida 107, however, low concentrations of hexadecyl-CoA appeared to stimulate activity of fatty acid synthetase complex, perhaps by helping to disperse the protein of the extract more thoroughly, and only when the ester exceeded 100 µM was inhibition observed (Fig. 2).

Activation and inhibition of acetyl-CoA carboxylase. The assay of acetyl-CoA carboxylase relies upon excess activity of the fatty acid synthetase complex within the crude extract; this was always present when assaying acetyl-CoA carboxylase in 100 mM-phosphate buffer (see Table 3) and therefore these assays were valid. When studying hexadecyl-CoA as an effector on acetyl-CoA carboxylase activity (see below), care was again taken to ensure that the activity of the fatty acid synthetase, though inhibited, was still above that of the carboxylase.

Citrate was found markedly to stimulate activity of acetyl-CoA carboxylase (Fig. 3). The response to stimulation by citrate, however, depended upon the state of Candida 107 from which the extract was obtained. If exponentially growing Candida 107 was used, a plot of enzyme activity against citrate concentration was sigmoidal (Fig. 3), but if the extract was prepared from yeast which had been starved for 24 h, the curve became hyperbolic though total activity was now decreased by about 65%. One explanation for this is that during the starvation period enzyme inhibitors, as well as the enzyme itself, were degraded within the
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Fig. 1. Activation of the fatty acid synthetase complex in extracts from glucose-grown Candida 107, starved for 24 h before harvesting, by fructose 1,6-diphosphate in the presence of either 0.1 M-phosphate buffer (pH 7.0) (), or 0.1 M-tris buffer ().

Fig. 2. Effect of palmitoyl-CoA on the fatty acid synthetase complex in extracts from glucose-grown Candida 107 starved for 24 h before harvesting.

yeast. Alternative explanations are however possible, as crude extract preparations were used in this work.

The effect of n-alkanes and their oxidation products (alcohol, free acid and acyl-CoA) on acetyl-CoA carboxylase activity was subsequently examined both in the presence and absence of citrate (Table I). n-Alkanes and hexadecanoic acid, up to 300 μM, had no effect. n-Decanol, however, stimulated activity both in the presence and absence of citrate, possibly by a similar mechanism to that previously noted for the stimulation of fatty acid synthetase activity by low concentrations of hexadecyl-CoA (see Fig. 2). Although 300 μM-hexadecyl-CoA was always strongly inhibitory, hexadecyl-CoA at 100 and 200 μM, and decanoic acid at 300 μM, strongly inhibited acetyl-CoA carboxylase activity only when assayed in the presence of citrate. The nature of the inhibitions brought about by these latter substances was therefore examined further.

Hexadecyl-CoA, at 100 and 200 μM, did not inhibit the basal activity of the enzyme but prevented the activation of the enzyme by citrate. There was a slow, linear increase in enzyme activity with increasing citrate concentration until, with the lower concentration of hexadecyl-CoA, a critical point was reached after which the increase in activity with increasing citrate concentration became extremely rapid (Fig. 4a). Presumably a similar pattern would have been observed with the higher concentration of hexadecyl-CoA if the concentration of citrate had been taken beyond 100 mM.

Decanoic acid produced a different pattern of inhibition to that brought about by hexadecyl-CoA, in that low concentrations of citrate continued to stimulate enzyme activity without any effect by added decanoic acid (Fig. 4b). Inhibition was only seen when activation by citrate had doubled the initial activity of the enzyme. Full activation was still attainable nevertheless by continuing to increase the citrate concentration. Dodecanoic acid had no effect on the enzyme activity at any concentration of citrate and is therefore, at 100 μM, equivalent in this respect to 300 μM-hexadecanoic acid.
Fig. 3. Citrate activation of acetyl-CoA carboxylase in extracts from glucose-grown *Candida* 107, (○) harvested at the end of exponential growth, or (■) starved for 24 h after the end of growth.

Table 1. *Action of possible effectors on activity of acetyl-CoA carboxylase from glucose-grown Candida* 107 harvested at the end of exponential growth

<table>
<thead>
<tr>
<th>Effector*</th>
<th>Conc. (μM)</th>
<th>Without citrate</th>
<th>Relative activity (%)</th>
<th>With citrate (75 μM)</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>—</td>
<td>5.2</td>
<td>100</td>
<td>31.6</td>
<td>100</td>
</tr>
<tr>
<td>Hexadecyl-CoA</td>
<td>100</td>
<td>5.6</td>
<td>108</td>
<td>31.6</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>5.6</td>
<td>108</td>
<td>9.2</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>0</td>
<td>0</td>
<td>1.8</td>
<td>6</td>
</tr>
<tr>
<td>Hexadecanoic acid</td>
<td>300</td>
<td>5.8</td>
<td>112</td>
<td>39.4</td>
<td>96</td>
</tr>
<tr>
<td>Decanoic acid</td>
<td>300</td>
<td>6.2</td>
<td>119</td>
<td>17.9</td>
<td>54</td>
</tr>
<tr>
<td>Decanol</td>
<td>500</td>
<td>7.2</td>
<td>138</td>
<td>54.6</td>
<td>171</td>
</tr>
<tr>
<td>n-Alkanes†</td>
<td>300</td>
<td>4.5</td>
<td>87</td>
<td>32.0</td>
<td>101</td>
</tr>
</tbody>
</table>

* Water-insoluble materials were added from a 5% (v/v) emulsion in water.
† A fraction of n-alkanes (C₁₉ to C₁₆) used.

Inhibition of enzyme activity by long-chain acyl-CoA esters was not confined to hexadecyl-CoA but was dependent upon the chain length of the acyl moiety (Table 2). This is similar to previous observations made with acetyl-CoA carboxylase isolated from rat liver (Bortz & Lynen, 1963). Dodecyl- and tetradecyl-derivatives slightly stimulated enzyme activity at concentrations that were inhibitory when the hexadecyl- and octadecyl-esters were used (Table 2).

*Alkane-grown Candida 107*

Repression of fatty acid biosynthesis. Extracts of *Candida* 107 grown on *n*-alkanes, or adapted to alkane utilization after growth on glucose, had activities of the fatty acid synthetase complex which were repressed to about half the activity observed in glucose-grown *Candida* 107. Even more striking was that the activity of the acetyl-CoA carboxylase had become almost completely repressed by exposure to alkanes (Table 3). The failure to detect
significant activity of this latter enzyme was not due to inability of these extracts to synthesize acetyl-CoA, for when acetyl-CoA was used in the assay system, no increase in the activity of the carboxylase was recorded (Table 3). The slight residual activity was independent of the presence of citrate (up to 200 mM was used) and unaffected by the addition of decanol or the absence of bicarbonate, though it did still show some slight decrease in activity when phosphate was omitted from the assay. These observations indicate that the apparent residual activity of this enzyme as measured is probably spurious, and due mainly to side-reactions involving formation of ether-soluble material, other than fatty acids, from acetate in the assay system. The actual repressed level of acetyl-CoA carboxylase, therefore, is probably much lower than those recorded in Table 3.
Table 3. Activities of acetyl-CoA carboxylase and the fatty acid synthetase complex of Candida 107 grown on glucose or alkanes

| Growth substrate | Growth conditions | Acetyl-CoA carboxylase for acetate (nmol converted/h/mg protein) | Fatty acid synthetase complex
<table>
<thead>
<tr>
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<tbody>
<tr>
<td>Glucose</td>
<td>Batch: harvested after 24 h</td>
<td>Acetate 31.6</td>
<td>70.0</td>
</tr>
<tr>
<td>Glucose</td>
<td>Batch: starved 24 h after glucose exhaustion</td>
<td>Acetyl-CoA 21.6</td>
<td>N.D.</td>
</tr>
<tr>
<td>C₁₃-C₁₆ alkanes</td>
<td>Continuous culture</td>
<td>Acetate 10.2</td>
<td>60.5</td>
</tr>
<tr>
<td>Glucose</td>
<td>Batch: adapted to hexadecane for 24 h after glucose exhaustion</td>
<td>Acetate 0.7</td>
<td>37.2</td>
</tr>
<tr>
<td>Glucose</td>
<td>Batch: adapted to decane for 24 h after glucose exhaustion</td>
<td>Acetyl-CoA 0.4</td>
<td>N.D.</td>
</tr>
<tr>
<td>Glucose</td>
<td>Batch: harvested after 24 h</td>
<td>Acetate 1.0</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

* Acetyl-CoA carboxylase activity measured in the presence of 75 mM-citrate.
N.D., not determined.

The absence of acetyl-CoA carboxylase activity in Candida 107 grown on alkanes could be due to an inhibitor within the extract. This was tested by mixing an extract from alkane-grown Candida 107 with an extract prepared from glucose-grown Candida 107 (Fig. 5). Although an inhibitor, or inhibitors, were indeed indicated to be present within the extract from alkane-grown Candida 107, these were not the cause of the lack of acetyl-CoA carboxylase activity, since in the mixed extract addition of 75 mM-citrate completely restored the activity to the expected value. There was no indication that any activity within this mixture was derived from the extract from alkane-grown Candida 107. The relief of inhibition by citrate followed the same pattern as that for the relief of acetyl-CoA carboxylase inhibited with hexadecyl-CoA (see Fig. 4a).

**DISCUSSION**

De novo fatty acid biosynthesis is absent when Candida 107 is grown on n-alkanes of any chain length. The enzyme which is principally regulated is acetyl-CoA carboxylase which is apparently totally repressed. Synthesis of this enzyme is clearly not co-ordinate with that of the fatty acid synthetase complex, whose individual genes in yeast are already known to be co-ordinately expressed (Schweizer, Kühn & Castorph, 1971), as only partial repression of the complex was observed even after several days’ continuous cultivation of the yeast on n-alkanes. The absence of carboxylase activity leads us to conclude that all fatty acids within the n-alkane-grown yeast must be derived from the n-alkanes themselves. This still applies when a short-chain alkane such as n-decane is used as sole carbon source and, with such substrates, elongation of the derived fatty acid must occur. The lipid of Candida 107 growing on an alkane with an odd number of carbon atoms, however, still contains a high proportion of even-chain-length acids (Thorpe & Ratledge, 1972) but there is no information regarding the mechanism by which these acids are formed. Some α-oxidation, as well as C₂-unit elongation, may be expected to occur. The absence of de novo fatty acid synthesis in alkan-
Fig. 5. Establishment of an inhibitor for acetyl-CoA carboxylase in extracts from alkane-grown Candida 107 which can be overcome by citrate. Activity of enzyme from glucose-grown Candida 107 starved before harvesting (○) and from alkane-grown Candida 107 (□). Activity of 1:1 mixture of both extracts: observed result (●); expected result if no interaction (----). (N.B. Activity of acetyl-CoA carboxylase in alkane-grown Candida 107 is still minimal even in the presence of high concentration of citrate; see text.)

grown Candida 107 suggests that this yeast may be useful for studying fatty acid modifications, particularly the poorly characterized C₁₄-unit elongation mechanism which occurs in many systems.

Acetyl-CoA carboxylase is not only a repressible enzyme but, being sensitive to allosteric effectors, is involved in the short-term, or fine, control of fatty acid biosynthesis. In this respect, the enzyme from Candida 107 appears similar to that obtained from other sources which are activated, with aggregation of monomer sub-units (Moss & Lane, 1972), by a number of metabolic intermediates, in particular, citrate (Lane, Moss, Ryder & Stoll, 1971). This activation can be competitively inhibited by long-chain fatty acyl-CoA esters (Numa, Ringlemann & Lynen, 1965; Goodridge, 1972) but it is still not clearly established that these effects constitute a control mechanism in vivo. The evidence relating to short-term control of acetyl-CoA carboxylase has been reviewed at length by Vagelos (1971), and doubts regarding the action of citrate and long-chain acyl-CoA esters in vivo apply equally to the results we have now obtained with Candida 107. More recently, however, Guynn et al. (1972), after an examination of possible effectors of fatty acid biosynthesis in freeze-clamped liver, concluded that only the concentration of long-chain fatty acyl-CoA ester varied in a manner which was consistent with that of a controlling metabolite.

The process of alkane oxidation clearly extensively modifies intermediary metabolism within Candida 107. Alkanes have already been shown to inhibit simultaneously both glucose transport and its catabolism (Gill & Ratledge, 1973a), and these events were conjectured to be instigated by products of alkane oxidation acting as direct feedback-inhibitors of certain
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key enzymes. Our results now indicate that of the intermediates of alkane oxidation, only long-chain fatty acyl-CoA esters, produced either directly from a long-chain alkane (C₁₆ to C₁₈ in length) or by elongation of a shorter acyl-CoA ester, could cause the inhibition of fatty acid biosynthesis. Such inhibition would lead to the accumulation of acetyl-CoA, and the consequences of this are likely to be profound as far as further effects on intermediary metabolism are concerned. Direct, or indirect, inhibition of glycolysis, glucose transport and other key processes can be anticipated to occur in keeping with the experimental observations. Since we can produce in Candida 107 either complete inhibition of glucose catabolism or the simultaneous utilization of both alkane and glucose, depending upon the state of the yeast and the chain length of the alkane substrate, this organism may be a useful model for studying the inter-relationships between fatty acid and carbohydrate metabolism including the exact nature of control of de novo fatty acid biosynthesis.

We gratefully thank Croda Universal Ltd, for a research scholarship in support of C.O.G.

REFERENCES


