Correlation of Lipid Accumulation in Yeasts with Possession of ATP: Citrate Lyase

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ATP: citrate lyase has been found in 13 strains of yeast (representing six genera) which are capable of accumulating lipid to above 20% of their biomass. The enzyme is absent in 10 other yeasts which do not accumulate lipid. The presence of the enzyme is therefore directly correlated to the phenomenon of oleaginicinity. The enzyme is located in the cytosol fraction of the yeasts and is probably the sole means of producing acetyl-CoA in most oleaginous yeasts. The specific activity of the enzyme correlates with the specific rate of lipid synthesis as determined in nitrogen-limited chemostat cultures of *Lipomyces starkeyi*, though not with the lipid content of the cells. From this and by calculation, it may be inferred that the enzyme is possibly the rate-limiting reaction for lipid biosynthesis.

INTRODUCTION

ATP: citrate lyase was first discovered in chicken liver (Srere & Lipmann, 1953), and it has subsequently been isolated from a variety of mammalian and plant sources (e.g. Hoffmann *et al.*, 1980a; Inoue *et al.*, 1966; Nelson & Rinne, 1975; Mattoo & Modi, 1970; Fritsch & Beevers, 1979). The enzyme, which is cytosolic, plays an important role in lipogenesis in many tissues by supplying the precursor acetyl-CoA units, which are themselves derived from intramitochondrial citrate (see Srere, 1975, for review). Some doubt, though, has recently been cast on the exact role of the enzyme in man (Hoffmann *et al.*, 1980b).

There have been few reports of the enzyme from microbial sources. It has been detected in the yeast *Rhodotorula gracilis* (Guerritore & Hanozet, 1970) and in the fungi *Mortierella* spp. (Attwood, 1973) and *Penicillium spiculispurum* (Mahlen, 1973). The first two reports described micro-organisms that are oleaginous, i.e. have the potential to accumulate a substantial proportion of their biomass as lipid; the other report concerned the biosynthesis of 2-decylcitric acid and 2-decylhomocitric acid, thus requiring (according to the author) large amounts of lauroyl-CoA to be synthesized. Some preliminary evidence was provided in each case that the pathway of lipogenesis was similar to that in plant and animal tissues and that increased rates of lipid biosynthesis could be correlated with enhanced ATP: citrate lyase activity. However, none of these studies was developed further.

In this laboratory we have been studying the biochemistry of microbial oleaginicinity and have advanced the hypothesis (Botham & Ratledge, 1979) that lipid accumulation in these organisms is a result of the concerted action of at least two separate metabolic events. Firstly, the NAD⁺-dependent isocitrate dehydrogenase of the mitochondrion has an absolute requirement for AMP so that when the AMP concentration is low, as occurs during nitrogen deprivation (which is needed to achieve lipid accumulation – see Ratledge, 1978, 1981), citric acid will accumulate. Secondly, and of possibly key importance, oleaginous micro-organisms possess ATP: citrate lyase which cleaves the citrate to acetyl-CoA (and oxaloacetic acid) so that fatty acid synthesis is constantly primed with substrate. This

hypothesis was advanced on the basis of a study of three oleaginous micro-organisms: two yeasts and one fungus.

In this present study we have sought to ascertain if the hypothesis is of general applicability and have examined 23 yeasts, both oleaginous and non-oleaginous, for the presence of ATP: citrate lyase. We have also evaluated the importance of phosphoketolases in lipid accumulation as these enzymes, which have been found in the oleaginous yeasts *Candida* 107, *Rhodotorula graminis* and *Rh. glutinis* (Whitworth & Ratledge, 1977), might provide an alternative route to ATP: citrate lyase for producing acetyl-CoA units in the cytosol. If phosphoketolases were ubiquitous amongst oleaginous micro-organisms their involvement may cast doubt on the necessity for ATP: citrate lyase.

METHODS

Growth of organisms. Yeasts were from either NCYC (Norwich, U.K.) or CBS (Baarn, The Netherlands), except *Candida curvata* strains R and D and *Trichosporon cutaneum* 40 which were the kind gift of Professor E. G. Hammond, Ames, Iowa, U.S.A. They were cultivated on a glucose/salts/yeast extract medium at pH 5.5, as previously described (Botham & Ratledge, 1979). Nitrogen-limited medium contained 30 g glucose l⁻¹ and 1.25 g ammonium tartrate l⁻¹; in carbon-limited medium these constituents were present at 10 g l⁻¹ and 1.5 g l⁻¹, respectively. Batch cultures were grown in 1 l vortex-aerated vessels, at 30 °C, without pH control (the pH rarely fell below pH 4.5). Cultures were harvested after 48 h. Continuous culture experiments were performed using a 5 l chemostat of conventional design. The basal salts/yeast extract media for the chemostat were the same as for batch culture except that the nitrogen source was NH₄Cl at 1 g l⁻¹ and glucose in the nitrogen-limited medium was at 100 g l⁻¹. Cultures were maintained at pH 5.5 by the automatic addition of NaOH. Other cultural conditions are described in Results.

Preparation of cell-free extracts. Cell-free extracts were prepared using a French press as previously described (Boulton & Ratledge, 1980). The suspending buffer was 50 mM-Tris/HCl, pH 7.5, containing 1 mM-MgCl₂ and 10 mM-dithiothreitol. In some experiments this was supplemented with 1 mM-ATP and 20 mM-tri-potassium citrate.

Enzyme assays. ATP: citrate lyase [EC 4.1.3.8; ATP: citrate oxaloacetate-lyase (CoA-acetylating and ATP-dephosphorylating)] was assayed at pH 8.3 according to Srere (1962) following the change in A_{340} due to the oxidation of NADH, when the oxaloacetate formed was reduced to malate by the action of malate dehydrogenase (EC 1.1.1.37). Phosphoketolases [EC 4.1.2.9; D-xylulose-5-phosphate D-glyceraldehyde-3-phosphate-lyase (phosphate-acetylating), and EC 4.1.2.22; D-fructose-6-phosphate D-erythrose-4-phosphate-lyase (phosphate-acetylating)] were assayed as described by Whitworth & Ratledge (1977). NAD⁺-dependent isocitrate dehydrogenase [EC 1.1.1.41; *threo*-D₂-isocitrate: NAD⁺ oxidoreductase (decarboxylating)] was assayed according to Kornberg (1955) measuring the change in A_{340} due to the reduction of NAD⁺. Citrate synthase [EC 4.1.3.7; citrate oxaloacetate-lyase (CoA-acetylating)] was assayed according to Srere *et al.* (1963), as previously described (Boulton & Ratledge, 1980). Glucose-6-phosphate dehydrogenase [EC 1.1.1.49; D-glucose-6-phosphate: NADP⁺ oxidoreductase] was assayed according to Noltmann *et al.* (1961) following the change in A_{340} due to the reduction of NADP⁺. Pyruvate dehydrogenase complex [EC 1.2.4.1; pyruvate: lipoamide oxidoreductase (decarboxylating and acceptor-acetylating), EC 1.6.4.3; dihydrolipoamide reductase (NAD⁺) (NADH: lipoamide oxidoreductase), and EC 2.3.1.12; dihydrolipoamide acetyltransferase (acetyl-CoA: dihydrolipoamide S-acetyltransferase)] was assayed by the method of Reed & Mukherjee (1969).

Protein estimation. Protein was assayed by the dye-binding procedure of Bradford (1976) using γ -globulin as standard (bovine, fraction II, Sigma).

Lipid estimation. Total lipid was estimated by a method based on that of Folch *et al.* (1957). Lipid was extracted by treating an accurately weighed sample of freeze-dried yeast (about 500 mg) with 150 ml chloroform/methanol (2:1, v/v) for 24 h. The mixture was filtered through Whatman no. 1 filter paper to remove cell debris and the filtrate was washed once with 30 ml distilled water. The extract was dried over anhydrous MgSO₄. After removal of the solvent by rotary evaporation, the lipid residue was taken up in diethyl ether and transferred to a tared vial. Solvent was removed in a stream of nitrogen and the lipid was dried in a vacuum desiccator and weighed.

Preparation of mitochondria. Yeast from 48 h vortex-aerated batch cultures cultivated on nitrogen-limited medium was harvested by centrifugation. After washing, the cell paste was suspended in 2 vol. of 50 mM-Tris/HCl, pH 7.5, containing 1.3 M-sorbitol, 1 mM-MgCl₂ and 10 mM-dithiothreitol. Yeast was disrupted by a single passage through a chilled French press at 35 MPa. Whole cells were removed by centrifugation at 2000 g and 4 °C for 10 min. The supernatant was carefully decanted and the mitochondrial and cytosolic fractions were separated by centrifuging at 19000 g and 4 °C for 20 min. The supernatant (cytosolic fraction) was retained and the crude

mitochondrial fraction (pellet) was washed by resuspension in the above medium and recentrifugation, as described. The mitochondrial fraction was resuspended in 2 vol. of the above buffer and mixed with 4 vol. of 100-mesh glass beads, and the mixture was homogenized for 1 min at 4 °C. The beads were removed by centrifugation at 2000 g for 5 min and the supernatant was retained.

RESULTS

Survey for the presence of ATP: citrate lyase in oleaginous and non-oleaginous yeasts

The ATP: citrate lyase activities of yeasts grown in batch culture are given in Table 1 along with the lipid content of the cells. The enzyme was present in all those yeasts capable of accumulating lipid to 20% (w/w) or more of their biomass. Maximum lipid accumulation occurred when nitrogen-limiting medium was used; with carbon-limiting medium, the lipid content was usually substantially lower but there was no decrease in ATP: citrate lyase activities under such conditions. The presence of the enzyme, therefore, indicates the potential of the cell for oleaginity though not its prevailing lipid content.

When a negative result for ATP: citrate lyase activity was obtained, assays were subsequently repeated both at pH 6.5 in 0.1 M-Na₂HPO₄/KH₂PO₄ buffer and at pH 7.5 in 0.1 M-Tris/HCl buffer but in no case was the original observation reversed. Dialysis of extracts from *Saccharomyces cerevisiae* and *Rhodospiridium toruloides* CBS 6016 against 0.05 M-Tris/HCl, pH 7.5 containing 1 mM-MgCl₂ and 10 mM-dithiothreitol also failed

Table 1. *Specific activities of ATP: citrate lyase and lipid contents of various yeasts*

Yeast	Carbon-limiting medium		Nitrogen-limiting medium	
	ATP: citrate lyase [nmol min ⁻¹ (mg protein) ⁻¹]	Lipid content (% dry wt)	ATP: citrate lyase [nmol min ⁻¹ (mg protein) ⁻¹]	Lipid content (% dry wt)
<i>Candida curvata</i> D	7.1	29.0	7.2	33.7
<i>Candida curvata</i> R	4.8	21.5	5.2	33.9
<i>Candida tropicalis</i> NCYC 4	0	2.3	0	4.0
<i>Candida utilis</i> NCYC 359	0	1.7	0	3.6
<i>Candida</i> 107 (=NCYC 911 = CBS 329.80)	6.1	18.9	7.8	25.1
<i>Cryptococcus albidus</i> NCYC 602	ND	ND	0	2.0
<i>Hansenula ciferrii</i> CBS 111	0	2.6	0	7.2
<i>Hansenula saturnus</i> CBS 5761	ND	ND	11.0	24.5
<i>Lipomyces lipofer</i> NCYC 944	ND	ND	50.0	36.2
<i>Lipomyces lipofer</i> CBS 5842	34.2	15.4	38.0	27.2
<i>Lipomyces lipofer</i> NCYC 692	ND	ND	0	1.5
<i>Lipomyces starkeyi</i> CBS 1809	37.2	21.2	54.0	36.5
<i>Lipomyces starkeyi</i> CBS 1807	ND	ND	19.0	29.8
<i>Lipomyces starkeyi</i> CBS 6132	0	2.8	0	6.4
<i>Lipomyces starkeyi</i> CBS 6047	37.0	38.5	51.0	42.5
<i>Rhodospiridium toruloides</i> CBS 6016	0	2.9	0	8.0
<i>Rhodospiridium toruloides</i> CBS 5490	48.6	3.9	41.8	25.9
<i>Rhodotorula glutinis</i> NCYC 59	9.0	15.8	12.0	23.7
<i>Rhodotorula graminis</i> NCYC 502	ND	ND	41.2	24.2
<i>Saccharomyces cerevisiae</i> NCYC 33	0	3.1	0	6.0
<i>Saccharomyces uvarum</i> NCYC 530	0	1.9	0	6.8
<i>Saccharomyces uvarum</i> NCYC 74	ND	ND	0	8.4
<i>Trichosporon cutaneum</i> 40	15.2	9.1	13.8	24.4

ND, Not determined.

Table 2. *Distribution of ATP: citrate lyase and other activities between cytosolic and mitochondrial fractions of Lipomyces starkeyi CBS 1809*

Enzyme	Expected location	Specific activity [nmol min ⁻¹ (mg protein) ⁻¹]	
		Mitochondrial fraction	Cytosolic fraction
NAD ⁺ -dependent isocitrate dehydrogenase	Mitochondrial	38	20
Citrate synthase	Mitochondrial	940	87
Pyruvate dehydrogenase	?	85	0
Glucose-6-phosphate dehydrogenase	Cytosolic	49	720
ATP: citrate lyase	?	0	34

to elicit activity, as did fractionation of the extracts by (NH₄)₂SO₄ precipitation. When extracts of ATP: citrate lyase-negative yeast were mixed with extracts containing the enzyme, no inhibition of the activity was observed indicating that activity of the enzyme was not being masked by the presence of an inhibitor. Preparation of cell-free extracts from *S. cerevisiae*, *S. uvarum* NCYC 530 and *Rsp. toruloides* CBS 6016 in buffers containing 1 mM-ATP and 20 mM-tri-potassium citrate, which stabilize the enzyme from *Lipomyces starkeyi* CBS 1809, also failed to produce any detectable activity. At least 50 separate attempts were made to detect activity of this enzyme in various non-oleaginous yeasts but all consistently gave negative results. The limit of detection of activity by the assay used was about 2–3 nmol min⁻¹ (mg protein)⁻¹.

Location of ATP: citrate lyase and pyruvate dehydrogenase in Lipomyces starkeyi CBS 1809

In previous work in this laboratory on the biochemistry of oleaginicinity in micro-organisms, *Candida* 107 was chosen as a representative yeast (e.g. Botham & Ratledge, 1979; Boulton & Ratledge, 1980). However, the activity of ATP: citrate lyase in this yeast is unusually low and it was decided that *Lipomyces starkeyi* CBS 1809 would be more suitable for subsequent work.

It is apparent that the sequence of events leading to lipid accumulation in these yeasts occurs partially as a result of intracellular compartmentalization. To confirm that the key enzymes are situated in the locations suggested, and to eliminate the possibility that acetyl-CoA units may be produced in oleaginous yeasts by a pyruvate dehydrogenase in the cytosol, a cell extract of *Lipomyces starkeyi* CBS 1809 was fractionated into a mitochondrial and a cytosolic fraction, as described in Methods, and each was assayed for pyruvate dehydrogenase and ATP: citrate lyase activities as well as marker enzymes of known location (Table 2). Allowing for some degree of mitochondrial damage during the preparation, the results indicate that pyruvate dehydrogenase is exclusively mitochondrial whilst ATP: citrate lyase is cytosolic. It follows, therefore, that the precursor acetyl-CoA for lipid biosynthesis is not derived directly from pyruvate in the cytosol. Instead, pyruvate enters the mitochondrion and is there converted to acetyl-CoA, as in other eukaryotes, followed by condensation with oxaloacetate to give citrate. The citrate may then be exported to provide the substrate for the cytosolic ATP: citrate lyase.

Phosphoketolase activity in oleaginous yeasts

ATP: citrate lyase activity appears to be closely linked to oleaginicinity in yeasts (and possibly fungi too) (Table 1). To consolidate our hypothesis that it is the key enzyme of lipid accumulation, it was necessary to ascertain if phosphoketolases, which already have been found in two species of oleaginous yeasts, *Rhodotorula graminis* and *Candida* 107 (Whitworth & Ratledge, 1977), were also of ubiquitous distribution in oleaginous yeasts. The

Fig. 1. Effect of dilution rate on lipid biogenesis and specific activity of ATP: citrate lyase in *Lipomyces starkeyi* CBS 1809 growing in a chemostat under nitrogen-limited conditions (a) and carbon-limited conditions (b). Activity of ATP: citrate lyase (O); lipid content of cells (Δ); biomass ($\bullet$); specific rate of lipid biosynthesis ($\blacktriangle$).

presence of these activities would result in the cytosolic production of acetyl-CoA directly from glucose and thus cast some doubt on the necessity for the ATP: citrate lyase pathway.

We confirmed the presence of phosphoketolase activity in *Candida* 107 and *Rhodotorula graminis* NCYC 502 [9.2 and 7.8 nmol min⁻¹ (mg protein)⁻¹, respectively]. However, extracts of *Hansenula saturnus* CBS 5761 and *Lipomyces starkeyi* CBS 1807 and CBS 1809 showed no activity of either hexose- or pentose-phosphoketolases. Thus, only in some oleaginous micro-organisms is ATP: citrate lyase supplemented by the activity of a phosphoketolase. Whether this latter enzyme makes a significant contribution to lipid biosynthesis is unknown, but clearly it is not essential for lipid accumulation to be achieved in an oleaginous yeast.

Effect of growth rate on the rate of lipogenesis and ATP: citrate lyase activity in chemostat cultures of Lipomyces starkeyi CBS 1809

In previous studies with *Candida* 107 and *Rh. graminis* in chemostat culture, the percentage of lipid in the biomass was higher at lower dilution rates (Gill *et al.*, 1977; Ratledge & Hall, 1979). If ATP: citrate lyase is regarded as the first enzyme of fatty acid biosynthesis in oleaginous micro-organisms, it may also be the rate-limiting step for the sequence, in which case its activity would probably be expected to vary with the rate of lipid synthesis. Attwood (1973) found that in species of *Mortierella* there was a positive correlation between the amount of lipid synthesized and ATP: citrate lyase activity, though rates of lipid synthesis were not determined. Similarly, the enzyme content of rat liver is known to vary with dietary changes and consequent alterations in rates of lipogenesis (Srere, 1975).

We therefore determined the activity of ATP: citrate lyase and the specific rate of lipid biosynthesis [expressed as g lipid synthesized h⁻¹ (g lipid-free yeast)⁻¹] at various growth rates on carbon-limiting and nitrogen-limiting media in chemostat cultures of *Lipomyces starkeyi* CBS 1809 (Fig. 1). Under nitrogen-limited conditions, the activity of ATP: citrate lyase correlated with the specific rate of lipid biosynthesis – as the latter increased with increasing growth rate (= dilution rate) so did the activity of the enzyme. There was no correlation of either of these with the lipid content of the cells. This, though, is not surprising as it is evident from these and other studies (see Gill *et al.*, 1977) that the amount of lipid

accumulated does not depend upon the rate of lipid synthesis increasing as the growth rate is lowered but rather on the synthesis of other macromolecules in the cell declining. Thus, at low growth rates lipid synthesis becomes a dominant aspect of the cell's metabolism.

Under carbon-limited conditions, ATP: citrate lyase remained as active as during nitrogen-limited growth indicating that it would not be limiting the rate of lipid biosynthesis. (It could therefore be argued that it would be the supply of citrate to the enzyme which would be the limiting factor for lipid biosynthesis under these conditions.)

DISCUSSION

An oleaginous micro-organism may be defined as one that has the potential to accumulate substantial amounts of lipid. Thus, it is implicit in the definition that high lipid contents might only be observed if the cultural conditions are such that lipogenesis is favoured. These conditions are met when there is an excess of carbon over some other nutrient, usually nitrogen, which results in a cessation of growth but allows the uptake of carbon to continue at an undiminished rate. Botham & Ratledge (1979) considered the lipid accumulation under these conditions in these organisms initially hinges on the build up of ATP and depletion of AMP. The intramitochondrial NAD^+ -dependent isocitrate dehydrogenase requires AMP for activity; thus, a stricture is imposed on the tricarboxylic acid cycle at this point. We have provided evidence that the activity of citrate synthase is not affected under these conditions (Boulton & Ratledge, 1980) and thus citrate is synthesized but not metabolized. The accumulating citrate is then exported to the cytosol in an exchange reaction, with an as yet unknown anti-porter (probably either malate or pyruvate), there to be cleaved by ATP: citrate lyase into acetyl-CoA and oxaloacetate, so providing the necessary substrate for lipid biosynthesis.

It follows therefore that the possession of ATP: citrate lyase would indicate a propensity for oleaginity and we consider our data now confirms this. The enzyme is the key to lipid accumulation as it is the only enzyme consistently found in all oleaginous organisms that would provide acetyl-CoA for fatty acid biosynthesis. Phosphoketolases, which might be an alternative route for acetyl-CoA synthesis, are of limited distribution in oleaginous yeasts and their contribution to provision of acetyl-CoA must be considered of minor importance. As far as we have been able to ascertain in these and previous studies there is probably nothing abnormal in the pathways of glucose metabolism or in the location of the various enzymes; thus acetyl-CoA is not produced from pyruvic acid in the cytosol as pyruvate dehydrogenase is intramitochondrial. The mere possession of ATP: citrate lyase does not, though, tell us anything about the organism's capacity for lipid storage. This must be determined by other factors.

No evidence for the presence of ATP: citrate lyase in non-oleaginous yeasts could be found. We presume that in these yeasts acetyl-CoA will be provided by acetyl-CoA synthetase acting on acetate derived from acetyl-CoA hydrolysis within the mitochondria (acetyl-CoA being unable to cross the mitochondrial membrane). One non-oleaginous yeast which we examined very carefully was *Saccharomyces uvarum* NCYC 74 (= *S. carlsbergensis* ATCC 9080) which was said by Hayashi *et al.* (1978) to contain ATP: citrate lyase activity. However, we were unable to substantiate this result and would in any case suggest that their values may be close to the limit of sensitivity of the assay used.

ATP: citrate lyase appears to be a constitutive enzyme in oleaginous yeasts as there was little significant difference in activity between cells from cultures in nitrogen-limited medium (which favours lipid accumulation) and those from carbon-limited medium. Nor did the activity change during the transition from carbon-limited growth to nitrogen-limited growth (C. A. Boulton, unpublished work). However, for nitrogen-limited growth of *Lipomyces starkeyi*, the changes, with growth rate, in the specific activity of the enzyme were similar to the changes observed in the specific rate of lipid biosynthesis (Fig. 1). This would be

consistent with the enzyme being the rate-limiting step of the entire pathway (as might be expected if ATP: citrate lyase were considered the first enzyme of lipid synthesis). Certainly the rate of the enzyme reaction is close to the overall rate of lipid synthesis, as indicated by the following calculation.

Taking the specific rate of lipid synthesis as $0.03 \text{ g lipid h}^{-1} (\text{g lipid-free yeast})^{-1}$, this is equivalent to $1.4 \text{ nmol lipid min}^{-1} (\text{mg protein})^{-1}$, assuming a protein content of 40% in the lipid-free yeast and the molecular weight of triacylglycerol as 890. As the ATP: citrate lyase must provide 27 mol acetyl-CoA to produce 1 mol triacylglycerol, the enzyme must be operating at a minimum rate of $1.4 \times 27 = 38 \text{ nmol acetyl-CoA produced min}^{-1} (\text{mg protein})^{-1}$. The observed $V_{\max}$ was between 60 and 70 $\text{nmol min}^{-1} (\text{mg protein})^{-1}$, at the lipid synthesis rate taken for this calculation, and so if the enzyme were catalysing the rate-limiting reaction, it would be operating at slightly over $\frac{1}{2}V_{\max}$, i.e. at about the K_m for the substrate.

This calculation again fits in with the enzyme possibly being the rate-limiting reaction of lipid biosynthesis in this yeast. Further work on the properties of the enzyme is currently in progress.

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