Activities of Regulatory Enzymes in Alkane-utilizing and Lipid-accumulating Yeasts and Moulds

By D. A. WHITWORTH AND C. RATLEDGE

Department of Biochemistry, The University, Hull HU6 7RX

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INTRODUCTION

Investigations with yeasts and moulds which accumulate up to 60% of their dry weight as lipid, mainly triglycerides, have been primarily concerned with the quantity and quality of lipid produced by different species. The few studies on the intermediary metabolism of these micro-organisms have been confined mainly to species of *Rhodotorula* and *Candida*. Brady & Chambliss (1967) and Höfer *et al.* (1969) demonstrated that phosphofructokinase was absent in all the species of *Rhodotorula* they examined. Barbalace, Chambliss & Brady (1971) showed that pyruvate kinase from *Rhodotorula glutinis* and other oxidative yeasts was not only insensitive to activation but its inhibition by ATP could not be relieved by fructose 1,6-diphosphate. The enzyme from *R. gracilis*, however, was activated by fructose 1,6-diphosphate (Höfer, Betz & Becker, 1970). Höfer *et al.* (1971) later showed that the pentose phosphate pathway was the predominant mechanism for carbohydrate metabolism in those yeasts lacking phosphofructokinase.

Observations with *Candida* 107, a lipid-accumulating, alkane-utilizing yeast, similar to *Rhodotorula* in possessing enzymes of the pentose pathway and lacking phosphofructokinase (Whitworth & Ratledge, 1975), led us to investigate whether the activities of regulatory enzymes of the pentose phosphate and glycolytic pathways are similar throughout lipid-accumulating or alkane-utilizing micro-organisms.

METHODS

Organisms and growth. All micro-organisms (see Table 1) were grown on glucose-limiting medium (Whitworth & Ratledge, 1975). All the strains of *Candida* are able to utilize alkanes but were not grown on them in this work. All the organisms except *Saccharomyces carlsbergensis*, *Candida lipolytica* and *C. tropicalis* accumulate lipid; lipid-accumulating organisms were selected from lists given in the reviews of Woodbine (1959) and Whitworth & Ratledge (1974). *Candida* 107 was grown in continuous culture at a dilution rate of 0.15 h^{-1} ; other yeasts were grown in shake flasks and harvested in the exponential phase (20 to 24 h). Moulds were grown in vortex-aerated, 1 l vessels and also harvested in the exponential phase (after 40 to 48 h). All organisms were grown at 30 °C except for the two species of *Rhodotorula* which were grown at 25 °C.

Preparation of cell-free extracts and enzyme assays. Organisms were harvested by centrifuging, then washed and resuspended in 50 mM-phosphate buffer pH 7.5 containing 1 mM-MgCl₂, to give 0.3 g packed mass/ml for yeast and 1.0 g/ml for mycelial fungi. Organisms were disrupted by a single passage through a French press at 35 MPa. The homogenate was centrifuged at 40000 g for 30 min and the precipitate discarded. The protein concentration of the supernatant solution was adjusted to 10 mg/ml. Enzymes assays (Whitworth &

Table 1. *Enzyme activities of some lipid-accumulating organisms*

Enzyme activities were determined on extracts after centrifuging at 40000 g for 30 min.
Values quoted are the averages of at least two determinations.

Micro-organisms	Enzyme activities (nmol/mg of protein/min)				
	Glucose 6-phosphate dehydrogenase	6-Phosphogluconate dehydrogenase	Phosphofructokinase	Fructose-1,6-disphosphatase	Pyruvate kinase
<i>Saccharomyces carlsbergensis</i> (NCYC530)	129	65	194	5	8
<i>Candida lipolytica</i> (ATCC8661)	290	161	194	13	323
<i>Candida lipolytica</i> (NCYC153)	565	403	400	16	323
<i>Candida tropicalis</i> (NCYC4)	290	129	0	13	581
<i>Candida</i> 107	516	516	0	10	194
<i>Hansenula anomola</i> (NCYC18)	290	226	323	10	215
<i>Rhodotorula glutinis</i> (NCYC59)	194	194	0	0	258
<i>Rhodotorula graminis</i> (NCYC502)	226	161	0	0	160
<i>Lipomyces starkeyi</i> (NCYC533)	161	194	226	3	20
<i>Geotrichum candidum</i> (IMI23312)	258	161	161	3	420
<i>Fusarium oxysporum</i> (IMI141140)	0	0	31	0	580
<i>Penicillium spinulosa</i> (IMI74803)	323	194	65	0	450
<i>Mucor javanicus</i> (IMI25330)	263	107	86	0	540

Ratledge, 1975) were completed within 7 h of harvesting the micro-organisms. All assays were carried out on at least two different preparations of cell extract. If there was a significant difference between these results, further assays were carried out to establish correct values.

Chemicals. All enzyme substrates and co-factors were from Sigma, except sodium citrate which was from Fisons Ltd, Loughborough.

RESULTS

Depending on the activity of the enzyme, normally 0.02 to 1 mg protein was used in 1 ml of each assay mixture. The absence of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in *Fusarium oxysporum* was confirmed with three different extract preparations and also by an examination of activity in the centrifuged particulate material. After determining the activities of all the enzymes in the absence of potential metabolic regulators (Table 1) we examined the effect of different concentrations of the following metabolites on each enzyme from all 13 organisms. The same preparation was used for all determinations to minimize variations in results.

Effect of NADPH. When added at concentrations greater than 0.6 to 0.7 mM, NADPH completely inhibited activity of all preparations of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. Its effect on the other enzymes was not investigated.

Effect of citrate. Citrate, at 10 mM, had little effect on glucose 6-phosphate dehydrogenase or 6-phosphogluconate dehydrogenase from any source and inhibition never exceeded 20 %. With phosphofructokinase, 10 mM-citrate usually resulted in up to 30 % inhibition, but in some lipid-accumulating micro-organisms strong inhibition was observed: the enzyme from *Hansenula anomola* was inhibited 80 % by 10 mM-citrate, and that from *F. oxysporum*, though only present at low concentrations, was completely inhibited by 2 mM-citrate.

Pyruvate kinase from the lipid-accumulating yeasts and moulds was more susceptible to citrate inhibition than that from other sources: with 10 mM-citrate, a 60 to 80 % decrease in activity occurred with the enzyme from *H. anomola*, *Rhodotorula graminis*, *Lipomyces starkeyi*, *Mucor javanicus* and *F. oxysporium*. However, the enzyme from the latter organism was, in fact, activated by 50 % with 2 mM-citrate. In the other micro-organisms there was little (< 20 %) or no inhibition with citrate up to 10 mM.

Effect of phosphoenolpyruvate. Glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were never inhibited by phosphoenolpyruvate by more than 20 to 30 %. The effect of this metabolite was not examined with the other enzymes.

Effect of ATP. ATP at 2 to 10 mM resulted in many extensive inhibitory effects. Although not strongly affecting glucose 6-phosphate dehydrogenase, 10 mM-ATP with 6-phosphogluconate dehydrogenase from both strains of *C. lipolytica*, *R. graminis*, *L. starkeyi* and *Penicillium spinulosa* resulted in 40 % inhibition. With *H. anomola*, 100 % inhibition was observed with ATP at this concentration. With phosphofructokinase from *C. lipolytica* ATCC8661, *Saccharomyces carlsbergensis*, *L. starkeyi*, *F. oxysporium* and *M. javanicus*, ATP at 2 mM produced 50 to 80 % inhibition, whilst similar inhibitions occurred with the same enzyme from *C. lipolytica* NCYC153 and *H. anomola* only with ATP at 10 mM. ATP did not appear to inhibit significantly the enzyme from *Geotrichium candidum* or *P. spinulosa*. With pyruvate kinase, ATP at 10 mM was normally inhibitory: it produced 40 % inhibition of the enzyme from both *Rhodotorulas*, 50 % inhibition with *H. anomola*, 60 % with *L. starkeyi* and *G. candidum*, and more than 90 % with *Candida* 107, *C. tropicalis*, both *C. lipolytica* strains and with *M. javanicus*. There was little inhibitory effect on the enzyme from *F. oxysporium*, *S. carlsbergensis* and *P. spinulosa*; however, 2 mM-ATP activated the enzyme from *F. oxysporium* by 50 %.

Effect of fructose 1,6-diphosphate. Pyruvate kinase, which was assayed at pH 7.5, was activated in all cases (except *Candida* 107 and both *Rhodotorula* species), with 5 mM-fructose 1,6-diphosphate by 20 to 30 %, though in *C. tropicalis* and *L. starkeyi* the enzyme was activated by 50 %. This effect could relieve, either completely or partially, the inhibition brought about by 10 mM-ATP.

DISCUSSION

Although our results are probably quantitatively different from those pertaining *in vivo*, they nevertheless should provide a guide as to the likely patterns of control with each organism. For alkane-utilizing micro-organisms no special common features could be distinguished, except possibly the occurrence of a slightly higher activity of fructose-1,6-bisphosphatase. This enzyme does indeed increase in activity following growth of *Candida* 107 on alkanes (Whitworth & Ratledge, 1975) although *n*-alkanes were never used as substrate in these present experiments. With lipid-accumulating micro-organisms there probably is the absence of some strategic regulatory point to allow the system to run 'out of control' and thus lead to an excessive accumulation of lipid. Although lipid production may be viewed as a means of disposing of excessive quantities of NAD(P)H, as in some bacteria producing poly- β -hydroxybutyrate (Senior & Dawes, 1971), this still leaves unanswered why such an excess of reducing power should be produced in the first place. With *Candida* 107 and the *Rhodotorula* species an 'out of control' situation is achieved by the absence of phosphofructokinase, which in other organisms is regulated by feedback inhibition by ATP, citrate and phosphoenolpyruvate. However, this enzyme was also absent from *C. tropicalis*, which is not a lipid-accumulating yeast and, moreover, was present in all the other lipid-accumulating micro-organisms examined. On the other hand, with *G. candidum* and

P. spinulosum this enzyme did not demonstrate allosteric inhibition by citrate or ATP and therefore would not be able to regulate glycolysis and the rate of entry of carbon into the acetyl-CoA pool and thence into fatty acids. How similar metabolic 'short circuits' are achieved in *H. anomala*, *L. starkeyi*, *F. oxysporium* and *M. javanicus* is not apparent. In these organisms other enzymes, not examined here, may serve to maintain intracellular concentrations of the potential feedback-inhibitors lower than were found to be effective in these *in vivo* assay systems.

The possession of an efficient pentose phosphate cycle, as the progenitor of NADPH, is also not a prerequisite for lipid accumulation. In *F. oxysporium*, which can accumulate 25 to 34 % of its cell dry weight as lipid (Woodbine, Gregory & Walker, 1951), the pentose phosphate cycle appeared completely absent. How this organism produces NADPH at a sufficiently rapid rate to sustain fatty acid synthesis is unknown. In all the other lipid-accumulating strains the pentose cycle, as manifested by the activities of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, was much in evidence, but so too was it in the other yeasts examined.

Thus, although all the organisms showed individual variations in the patterns of control over glycolysis, it was not always possible to explain why a particular species was, or was not, capable of lipid accumulation. With *Candida* 107, the *Rhodotorula* species, *G. candidum* and *P. spinulosa*, the reasons are perhaps obvious. Further work is needed with the other organisms, however, before our proposal that lipid-accumulation depends upon a strategic control point being absent, can be considered of general applicability.

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