Phosphofructokinase and the Regulation of the Flux of Carbon from Glucose to Lipid in the Oleaginous Yeast *Rhodospiridium toruloides*

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Changes in cell composition of *Rhodospiridium toruloides* CBS 14 were monitored during growth of batch cultures with NH_4Cl and glutamate as nitrogen sources. Carbohydrate was synthesized at the expense of lipid in NH_4^+ -grown cells, whereas in glutamate-grown cells lipid accumulation was predominant. Total biomass and protein concentration were similar in both cultures. Uptake of $[\text{U-}^{14}\text{C}]$, $[1\text{-}^{14}\text{C}]$ and $[6\text{-}^{14}\text{C}]$ glucose, and evolution of $^{14}\text{CO}_2$ from these sources, by washed suspensions of cells grown on glutamate revealed the flux of carbon during glucose dissimilation was principally via the Embden–Meyerhof pathway (72%), with 28% being metabolized by the pentose phosphate pathway. Both urea and glutamate, when added to the cell suspensions, significantly stimulated glucose catabolism, with the flux of carbon via the former pathway increasing to about 89% of the total. Phosphofructokinase (PFK) was implicated as the likely controlling enzyme to explain these events.

PFK was only detected in extracts prepared from the yeast grown in a carbon-limited (nitrogen-excess) medium; no activity was detected in extracts of cells grown in nitrogen-limited medium. The presence of a protease in these latter extracts was revealed. PFK was purified 92-fold to a final specific activity (in the presence of 10 mM- NH_4^+) of 4.2 units (mg protein) $^{-1}$ and exhibited one broad band on polyacrylamide gel electrophoresis. The apparent mol. wt (M_r) of the enzyme was approx. 700000. The major properties of the enzyme were examined to determine its regulatory role during lipid biosynthesis. Unlike the enzyme from *Saccharomyces cerevisiae*, no inhibition was found with 10 mM-ATP. ADP was not inhibitory either. NH_4^+ ions increased activity 11-fold by increasing the affinity of the enzyme for both fructose 6-phosphate and ATP. K^+ ions also stimulated activity but to a lesser extent. Activity was severely inhibited by citrate, isocitrate and *cis*-aconitate but this inhibition was dramatically alleviated by NH_4^+ . Inhibition by citrate was competitive with fructose 6-phosphate in the absence of NH_4^+ ions. The K_i values for citrate were 1.0 mM (with no NH_4^+) and 7.2 mM (with 10 mM- NH_4^+). Long-chain fatty acyl-CoA esters had no significant inhibitory effect. It is concluded that the interplay between the prevailing intracellular concentrations of NH_4^+ and citrate is the major determinant of the activity of PFK *in vivo* and thus governs the extent to which glucose is converted either to lipid or carbohydrate.

INTRODUCTION

Until recently, investigations into the control of lipid accumulation by yeasts have compared various aspects of metabolism in oleaginous yeasts with those in non-oleaginous species (Botham & Ratledge, 1979; Boulton & Ratledge, 1981). However, recently we have examined the effect of the medium nitrogen source on lipid accumulation, as it had previously been reported that certain yeasts could accumulate substantially more lipid when various organic nitrogen sources, rather than NH_4^+ , were used for growth (see for example Blinc & Hočevár, 1953;

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Abbreviations: FACE, fatty acyl-coenzyme A ester; F6P, fructose 6-phosphate; PFK, phosphofructokinase.

Witter *et al.*, 1974). By screening numerous yeasts for lipid accumulation with several different nitrogen sources, we were able to confirm these early reports and identify a small number of yeasts that could produce different amounts of lipid depending on the nitrogen source (Evans & Ratledge, 1984*a*). We chose the yeast *Rhodospiridium toruloides* CBS 14 for all subsequent work, as its lipid content was shown to vary from 18% (w/w), when grown with NH_4^+ as nitrogen source, to 52% (w/w) when glutamate or urea was used (Evans & Ratledge, 1984*a, b*).

This yeast therefore provided us with a system of lipid accumulation in which the lipid content could be controlled by just one factor, namely the nature of the nitrogen source used in the medium. We have shown, using this yeast, that nitrogen metabolism and lipid biosynthesis are intimately linked. The production of NH_4^+ ions intracellularly, as a result of catabolism of the nitrogen source, correlates well with the increased synthesis of citric acid (the precursor of the cytosolic acetyl-CoA essential for lipid production) (Evans & Ratledge, 1984*a, b*). In an attempt to determine the nature of the interaction of the products of nitrogen catabolism with lipid biosynthesis, we have examined the influence of the nitrogen source on the synthesis of major cellular components, and from this have gone on to investigate the effect of the nitrogen sources on the flux of carbon through the two major pathways of glucose dissimilation: the Embden-Meyerhof pathway and the pentose phosphate pathway, which are known to be the predominant pathways for glucose metabolism in this yeast (Mazon *et al.*, 1974; Ratledge & Botham, 1977). The results we obtained suggested that phosphofructokinase (PFK) could play a key role in regulating the flow of carbon through the glycolytic pathway with its activity *in vivo* being controlled by the choice of nitrogen source used in the growth medium.

PFK is, of course, well-established as a key regulatory enzyme in almost all organisms, including yeasts (Fraenkel, 1982; Uyeda, 1979). We therefore undertook the purification and study of the properties of this enzyme from *Rs. toruloides*. The enzyme was originally thought to be absent in this yeast (syn. *Rhodotorula glutinis*) (Höfer *et al.*, 1971) but was subsequently identified using carefully controlled conditions of assay (Mazon *et al.*, 1974). In the course of this work we have found that there is a protease, possibly specific for PFK hydrolysis, within the yeast which could account for the lability of previous preparations.

METHODS

Yeast and growth conditions. *Rhodospiridium toruloides* CBS 14 was grown on the basal medium as previously described by Evans & Ratledge (1984*a*) with glucose at 30 g l^{-1} and NH_4Cl at 0.5 g l^{-1} for nitrogen-limited medium, and with glucose at 12 g l^{-1} and NH_4Cl at 3.0 g l^{-1} for carbon-limited medium.

Analyses. Biomass and lipid were determined by procedures given previously (Evans & Ratledge, 1983). Total cell protein was determined using the biuret method as described by Herbert *et al.* (1971) using 5 mg freeze-dried cells suspended in 0.5 ml water. Total cell carbohydrate was determined by the phenol/ H_2SO_4 method described by Herbert *et al.* (1971) using 10 mg freeze-dried cells (20 to 100 μg glucose equivalents).

Determination of individual polysaccharides. Trehalose, glucan and mannan were determined by the method of Trevelyan & Harrison (1956) using 5% phenol. Glycogen was determined using amyloglucosidase by the method of Gunja-Smith *et al.* (1977). Intracellular extracts for metabolite assay were prepared as described previously (Evans & Ratledge, 1984*a*).

Incorporation of radioactivity. Uptake of $[\text{U}-^{14}\text{C}]$, $[1-^{14}\text{C}]$ and $[6-^{14}\text{C}]$ glucose (Amersham) into lipid and evolution of $^{14}\text{CO}_2$ by cell suspensions of *Rs. toruloides* was followed using the procedure described by Ratledge & Botham (1977). Cells for the experiment had been grown on glucose/glutamate medium for 30 h. Incubations, at 30°C , were started by adding 200 μl of radiolabelled glucose solution, containing 3 mg glucose with 3 μCi (111 kBq) activity, into each sealed Warburg flask. One flask was used for each separate determination with the reaction being stopped by adding H_2SO_4 from the side-arm of the flask. Suspensions were neutralized with KOH before freeze-drying and extracting the lipid. The radioactivity in the lipid and the filter paper, placed in the central well of the flask to trap $^{14}\text{CO}_2$, was counted by liquid scintillation. The percentage carbon flux through the pentose phosphate cycle and, by difference, the amount through the Embden-Meyerhof pathway, was calculated using the formula of Wood *et al.* (1963):

$$\frac{^{14}\text{C in triose phosphate derivative (i.e. lipid) from } [1-^{14}\text{C}]\text{glucose}}{^{14}\text{C in triose phosphate derivative from } [6-^{14}\text{C}]\text{glucose}} = \frac{1 - \text{PC}}{1 + 2\text{PC}}$$

where PC = pentose phosphate cycle.

Preparation of cell extracts. Cells were washed twice with 100 mM-KH₂PO₄/Na₂HPO₄ buffer, pH 7.8, and were usually disrupted by a single passage through a pre-cooled French pressure cell at 35 MPa (cf. Evans & Ratledge, 1983). Cells were also disrupted ultrasonically (cf. Hughes *et al.*, 1971) or by forming sphaeroplasts with Zymolyase 6000 (Miles Laboratories, Stoke Poges, Bucks., UK) which were then subsequently broken by gentle shaking. Cells were also lysed with toluene (Serrano *et al.*, 1973) in order to assay phosphofructokinase *in situ* (cf. Ratledge & Botham, 1977).

Determination of protein concentration. This was done by the dye-binding method of Bradford (1976) using bovine γ -globulin as standard.

Enzyme assay. 6-Phosphofructokinase (EC 2.7.1.11) (PFK) was assayed by the method of Sols & Salas (1966) using the coupled enzyme spectrophotometric procedure with ATP as co-substrate and potassium phosphate buffer at pH 8.0. The coupling enzymes (aldolase, triosephosphate isomerase and α -glycerophosphate dehydrogenase) were desalted to remove (NH₄)₂SO₄, which is a positive effector of PFK, by dialysis before use.

Purification of PFK. All procedures were carried out at 4 °C. The yeast was cultivated on carbon-limiting medium and washed in an extraction buffer containing 100 mM-KH₂PO₄/Na₂HPO₄ pH 7.8, 100 mM-KCl, 10 mM-mercaptoethanol, 1 mM-fructose 6-phosphate and 100 mg phenylmethylsulphonyl fluoride (PMSF) l⁻¹ as a protease inhibitor. The cell-free extract was prepared by a single passage through a pre-cooled French pressure cell at 35 MPa and centrifuged at 43000 g for 30 min.

A freshly prepared suspension of protamine sulphate (1.5%, w/v), adjusted to pH 6.4 with KOH, was added to the crude extract to give a final ratio of 1 mg protamine sulphate to 12 mg protein. The precipitate was then removed by centrifugation at 30000 g for 20 min and discarded. Fractionation with (NH₄)₂SO₄ was carried out, collecting the fraction precipitating between 39 and 49% saturation. This precipitate was collected and dissolved in buffer (no. 2) containing: 50 mM-KH₂PO₄/Na₂HPO₄ pH 7.8, 5 mM-MgCl₂, 1 mM-EDTA, 5 mM-mercaptoethanol, 1 mM-fructose 6-phosphate and 100 mg l⁻¹ PMSF. The suspension was subjected to a second (NH₄)₂SO₄ fractionating, collecting the fraction precipitating between 44 and 53% saturation. The precipitate was collected and dissolved in buffer no. 2.

The enzyme solution was applied to a column of Sephadex G-200 (2.5 × 38 cm) equilibrated with buffer no. 2. After elution with the same buffer, the fractions containing PFK activity were pooled and eluate applied directly to a column of DEAE-cellulose (DE-52) (2.5 × 35 cm) equilibrated in buffer no. 2. The column was eluted with the same buffer until the eluate was free of protein (monitored at 280 nm).

A continuous gradient of KCl (0 to 300 mM) in buffer no. 2 was used to elute the enzyme which appeared between 40 to 80 mM-KCl. This fraction was pooled, and solid (NH₄)₂SO₄ added to give 50% saturation. The precipitate was dissolved in 5 ml buffer no. 2 and applied to a column of Sepharose-6B (3.5 × 50 cm) equilibrated with buffer no. 2. The elution profile showed a single main protein peak which contained all the PFK activity. The pooled Sepharose-6B eluate was concentrated over an Amicon PM10 membrane to a 5 ml volume and stored at -20 °C in 10 mM-(NH₄)₂SO₄ and 1 mM-fructose 6-phosphate.

Determination of purity of PFK preparation. Samples of the concentrated enzyme, containing approximately 50 to 100 μ g protein, were developed at 4 °C on 5% (w/v) polyacrylamide gels, according to the procedure of Davis (1964) in 0.04 M-Tris/glycine buffer, pH 8.3 at 5 mA per tube. Protein was detected by staining for 3 h in Serva Blue G (1% Serva Blue G in 12% TCA; Serva Feinbiochemica, Heidelberg, FRG). No destaining was necessary.

Calibration of Sepharose-6B column for determination of molecular size of PFK. A column of Sepharose-6B (3.5 × 50 cm) was equilibrated with 50 mM-KH₂PO₄/Na₂HPO₄ buffer pH 7.8. The void volume of the column was determined using blue dextran (Pharmacia). The following proteins were loaded on to the column in a total volume of 2.8 ml and eluted in the above buffer: urease, β -galactosidase, arginine decarboxylase, pyruvate kinase, aldolase and malate dehydrogenase. Thyroglobulin was run separately and assayed spectrophotometrically at 280 nm. The other proteins were detected by appropriate enzyme assays.

RESULTS

Change in cell composition during growth of Rs. toruloides in batch culture

When *Rs. toruloides* CBS 14 had been previously grown on glutamate as nitrogen source, the lipid content increased to over 50% of the biomass, compared to less than 20% when NH₄Cl had been used (Evans & Ratledge, 1984a). However, the NH₄⁺-grown cells still assimilated glucose and grew to the same yield as the glutamate grown cells; a detailed analysis of lipid, carbohydrate and protein contents of the two types of culture was therefore carried out to discover the nature of the material which these cells must be accumulating instead of lipid (Fig. 1).

During the first 30 h growth, that is up to the exhaustion of the nitrogen source from the medium, cells of both cultures had the same gross composition. However, after 30 h there was a marked divergence in accumulation of carbohydrate and lipid though the protein content of the

Fig. 1. Changes in cell composition of *Rs. toruloides* CBS 14 during batch culture with growth on (a) NH₄Cl and (b) glutamate as nitrogen sources. ■, Biomass; ○, carbohydrate; ●, protein; □, lipid. Nitrogen (not shown) was exhausted from both media after 24 to 28 h.

two cultures throughout growth was almost the same: at the end of growth (90 h) NH₄⁺-grown cells contained 23% protein, whereas glutamate-grown cells had 20%. The carbohydrate and lipid contents showed a strong, reciprocal relationship between the two types of cells with the NH₄⁺-grown cells finally having over twice the carbohydrate (42% compared to 20%) but less than half the lipid (21% compared to 51%) of the glutamate-grown cells. These results suggested there might be an interplay between the lipid and carbohydrate synthesizing systems, viz:

Any block in the synthesis of one component would then divert the incoming carbohydrate into the other. The existence of such an interplay would then fit in with the previously observed rise in the concentration of glucose 6-phosphate in NH₄⁺-grown cells where, after 60 h growth, the concentration was twice that in glutamate-grown cells (Evans & Ratledge, 1984a).

The additional (accumulating) carbohydrate in the NH₄⁺-grown cells appeared to be mainly glycogen – both acid- and alkali-soluble. These cells contained 26% glycogen compared to only 11% in glutamate-grown cells; trehalose content was approximately the same in both types of cell (6.6% and 6.2%) with mannan plus glucan accounting for 5.6% and 2.2%, respectively.

If this view of a switch in metabolism were correct, we would expect to see changes in the flux of carbon to lipid via the major pathways of metabolism: the Embden–Meyerhof pathway and the pentose phosphate pathway, according to the nature of the nitrogen source which was used.

Table 1. *Incorporation of radiolabelled glucose into lipid, and $^{14}\text{CO}_2$ evolution by cell suspensions of *Rs. toruloides**

See Methods for incubation conditions. Each line represents a single flask.

Nitrogen source (20 mM) added to incubation mixture	Radioactivity incorporated from:						% Flux through†:	
	[U- ^{14}C]Glucose		[1- ^{14}C]Glucose		[6- ^{14}C]Glucose		PP pathway	EM pathway
	Lipid*	$^{14}\text{CO}_2$ †	Lipid*	$^{14}\text{CO}_2$ †	Lipid*	$^{14}\text{CO}_2$ †		
None	1508	420	979	280	2152	40	28	72
None	1083	462	1099	295	2594	62	31	69
NH_4Cl	994	497	1132	309	2490	21	29	71
NH_4Cl	1348	483	1309	314	2542	39	24	76
L-Glutamate	2687	747	2243	465	3072	109	11	89
L-Glutamate	2605	799	2112	394	3019	83	13	87
L-Glutamate	2533	712	2898	378	3683	88	8	92
L-Glutamate	1227	694	1982	402	2804	94	12	88
Urea	1979	637	3004	398	4243	122	12	88
Urea	2853	702	2529	377	3416	97	10	90

* c.p.m. per mg lipid.

† c.p.m. released per incubation.

‡ Calculated according to Wood *et al.* (1963), see Methods. PP, pentose phosphate; EM, Embden-Meyerhof.

We therefore investigated the effect of glutamate and NH_4^+ upon the pathways of glucose catabolism and of lipogenesis in glutamate-grown cells (Table 1). Glutamate-grown cells were used in preference to NH_4^+ -grown cells as they possess an active NAD^+ -glutamate dehydrogenase which is repressed in the latter cells (Evans & Ratledge, 1984*a*). Addition of glutamate to cell suspensions of NH_4^+ -grown cells would therefore have little immediate effect on lipogenesis or on the fluxes of glucose metabolism as it is, in all probability, the prevailing intracellular concentration of NH_4^+ which is the important metabolic regulator, rather than glutamate *per se*. Addition of glutamate (or urea) to glutamate-grown cells, on the other hand, would quickly lead to increases in the intracellular NH_4^+ concentrations. The incorporation of ^{14}C into lipid was measured as this gives an indirect measure of the formation of acetyl-CoA as the end-product of glucose catabolism from which the proportion of glucose metabolized by the pentose phosphate pathway can be calculated (see Wood *et al.*, 1963). Evolution of $^{14}\text{CO}_2$ was also followed. However, it is not possible to calculate the percentage contribution of the pentose phosphate pathway from such data unless the specific yield of $^{14}\text{CO}_2$ per amount glucose utilized can be determined (Wood *et al.*, 1963) and this was not done. Comparison of the $^{14}\text{CO}_2$ evolution values nevertheless provide a qualitative guide as to changes in the amount of glucose being metabolized by both the pentose phosphate and the Embden-Meyerhof pathways.

The incorporation of ^{14}C into lipid from [U- ^{14}C]glucose was only slightly higher than that from [1- ^{14}C]glucose but both were substantially less than that from [6- ^{14}C]glucose. Rates of incorporation, measured over the 60 min incubation (data not shown), were reasonably constant. Addition of NH_4^+ gave no increase in the incorporation of ^{14}C from any of the three labelled glucoses. Glutamate, and also urea, when added to the cell suspensions, produced large increases with all three labelled glucoses, with the effect being strongest with both [U- ^{14}C] and [1- ^{14}C]glucose. Again, the increases caused by glutamate and urea were reasonably constant over the whole of the incubation period (not shown). The proportion of glucose being metabolized via the Embden-Meyerhof pathway in the presence of glutamate or urea was calculated as between 87 and 92%, compared with between 69 and 76% in their absence or in the presence of NH_4^+ (Table 1).

Evolution of $^{14}\text{CO}_2$ from the three labelled glucoses also showed reasonably constant rates over 60 min incubation (data not shown). Analysis after 60 min (Table 1) showed that NH_4^+ produced little, if any, significant increase and, with [6- ^{14}C]glucose, appeared, if anything, to depress $^{14}\text{CO}_2$ evolution slightly. Both glutamate and urea stimulated the evolution of $^{14}\text{CO}_2$ from

Table 2. *Purification of phosphofructokinase from Rs. toruloides CBS 14*

Step	Total activity ($\mu\text{mol min}^{-1}$)	Total protein (mg)	Specific activity ($\text{nmol mg}^{-1} \text{min}^{-1}$)	Recovery (%)	Purification (fold)
Crude	94.95	1899	50	100	1
Protamine sulphate	81.84	880	93	86	1.8
$(\text{NH}_4)_2\text{SO}_4$ precipitation (39–49%)	53.16	194	274	56	5.4
$(\text{NH}_4)_2\text{SO}_4$ precipitation (44–53%)	45.9	58	792	48	15.9
Sephadex G-200	42.2	30	1408	44	28.2
DEAE-cellulose (DE-52)	34.9	12	2917	35	58.3
Sepharose 6B	27.6	6	4619	28	92.4

all three labelled glucoses, indicating that there was an increase in the total amount of glucose now being catabolized by the two pathways. The figures for $^{14}\text{CO}_2$ evolution from $[\text{U-}^{14}\text{C}]$ -glucose would indicate that this increase could be as much as 70%; the figures for $^{14}\text{CO}_2$ release from $[6\text{-}^{14}\text{C}]$ glucose, where the radiolabel would not be lost until after the level of pyruvate had been reached, would support this estimate.

We would interpret these results as follows. Growth of *Rs. toruloides* on glucose with NH_4^+ as nitrogen source has a restricted flow of carbon through both the Embden–Meyerhof and pentose phosphate pathways. Consequently, incoming glucose is channelled more into reserve carbohydrates than into lipid even though this yeast has the necessary metabolic machinery to effect lipid accumulation. In the presence of an organic source of nitrogen (see Evans & Ratledge, 1984*b*), such as glutamate or urea, the metabolic impediment to glucose catabolism is removed and more glucose is now able to reach the level of citrate and hence acetyl-CoA and lipid. As this increase in throughput of glucose to lipid corresponds with the previously noted depletion in glucose 6-phosphate concentration (Evans & Ratledge, 1984*a*) and, with an increased percentage of glucose now flowing through the Embden–Meyerhof pathway (Table 1), it seems reasonable to suppose that the principal controlling enzyme would be PFK. Previous studies (Evans & Ratledge, 1984*a, b*) have shown that the intracellular concentrations of NH_4^+ are highest when organic compounds are used as nitrogen sources and are lowest when NH_4^+ salts are used. As the controlling influence of NH_4^+ on the activity of PFK in other yeasts is well-established (Sols *et al.*, 1971; Uyeda, 1979) the importance of this enzyme in *Rs. toruloides* was investigated.

PFK in crude extracts

Despite numerous attempts to stabilize it, only very low activity of PFK could be detected in crude extracts prepared by a variety of means (see Methods) from cells grown in nitrogen-limiting (lipid accumulating) medium. Such lability had also been encountered by Mazon *et al.* (1974), and in another oleaginous yeast, *Candida* 107, by Ratledge & Botham (1977), who finally used yeast cells made permeable with toluene to demonstrate PFK activity. Toluene-permeabilization also worked with *Rs. toruloides* but subsequent disruption of these cells led to a rapid loss of activity. However, activity could be detected in extracts of *Rs. toruloides* grown on carbon-limiting medium. When extracts from these cells were mixed (1:1) with ones from cells grown in nitrogen-limiting medium, PFK activity was lost within 30 min incubation at 30 °C. This inactivation was prevented by adding PMSF as an inhibitor of proteolytic activity (Holzer *et al.*, 1975). The protease present in the nitrogen-limited extract appeared to be somewhat specific for PFK as activity of other large molecular weight enzymes (ATP: citrate lyase, NAD^+ -isocitrate dehydrogenase and aldolase) from this yeast were not affected.

Purification of phosphofructokinase

PMSF was used as protease inhibitor in all buffers throughout the purification. The procedure used for purifying PFK (Table 2) gave a 92-fold purification with a 28% recovery. PFK eluted as a single sharp peak of activity following gel filtration through Sepharose 6B. When 50 μl of this preparation was subjected to polyacrylamide disc gel electrophoresis and stained with Serva Blue for 3 h, only a single band of protein was detected; after a further 7 h staining, three very

Fig. 2

Fig. 2. Effect of F6P concentration on the activity of purified PFK. Assay contained ATP at 1 mM. ●, No NH_4^+ ; □, 4 mM- NH_4^+ ; ○, 10 mM- NH_4^+ .

Fig. 3

Fig. 3. Stimulation of purified PFK activity by NH_4^+ ions at varying concentrations of F6P. Assay contained 1 mM-ATP. ●, 0.5 mM-F6P; ○, 1 mM-F6P; ■, 5 mM-F6P.

faint bands emerged, one close to the main band and two some distance away. These faint bands could be either contaminant proteins or sub-units of the large PFK molecule.

The apparent molecular weight (M_r) of PFK from *Rs. toruloides* was determined by passing a sample of the purified enzyme down a column of Sepharose 6B and comparing the elution volume with the elution volumes of standards of known molecular weight (see Methods). A plot of log mol. wt against the ratio of the elution volume to the void volume gave an apparent molecular weight (M_r) of 700000.

The purified preparation was stable for up to 5 d at -20°C but lost 42% activity after 6 h at 0°C . PFK could be stabilized by adding ATP (1 mM), F6P (1 mM) and $(\text{NH}_4)_2\text{SO}_4$ (10 mM), either singly or in combination, and was stored at -20°C in 10 mM- $(\text{NH}_4)_2\text{SO}_4$ and 1 mM-F6P for up to 3 months without significant loss of activity. All preparations were desalted by passing down a column of Sephadex G-25 prior to studying the kinetic properties.

Properties of the purified enzyme preparation

The activity of PFK was stimulated by NH_4^+ and so all subsequent experiments were performed both in the presence and absence of NH_4^+ ions. The enzyme exhibited maximum activity between pH 7.4 and 8.2 in 0.1 M-phosphate buffer; activity fell by 50% at pH 6.5 and by 33% at pH 8.5. A pH of 8.0 was used in all subsequent assays.

In the presence of 10 mM- NH_4^+ , the V_{max} of PFK was increased from 1.02 to 4.23 $\mu\text{mol mg}^{-1} \text{min}^{-1}$ (Fig. 2). The apparent K_m , calculated by the usual double reciprocal plot, for F6P in the absence of NH_4^+ ions was 1.25 mM whereas NH_4^+ ions increased the affinity of PFK for F6P with a K_m of 0.55 mM. This conclusion was confirmed by following the effect of NH_4^+ ions on PFK activity at three concentrations of F6P (Fig. 3): with 10 mM- NH_4^+ , the activity of PFK was increased 4-fold with 5 mM-F6P, 5-fold with 1 mM-F6P and 11-fold with 0.5 mM-F6P.

The apparent K_m for ATP in the absence of NH_4^+ ions was calculated as 0.41 mM and to be 0.29 mM in the presence of 10 mM- NH_4^+ . In comparison with the effect of NH_4^+ ions on the affinity of PFK for its other substrate, F6P, this is a much smaller, and perhaps insignificant, effect. Maximum activity, with or without NH_4^+ , was obtained at 1 mM-ATP; no inhibition of activity occurred at 10 times this concentration.

Effect of nucleotides

Nucleotide triphosphates other than ATP could also function in the PFK reaction though to a lesser extent than ATP (Table 3). Apparent K_m values, determined in the usual manner, in the absence of NH_4^+ ions, were 0.61 mM for GTP, 0.69 mM for ITP, 0.83 mM for CTP and 1.36 mM

Fig. 4. Effect of energy charge on the activity of purified PFK. Assay contained 5 mM-F6P and 10 mM- NH_4^+ . Energy charge values were calculated according to Bomsel & Pradet (1968). Total adenine nucleotide concentrations: Δ , 0.1 mM; $\bullet$, 1 mM; $\circ$, 10 mM.

Table 3. Activity of purified PFK with various nucleotides

The standard assay was used containing 5 mM-fructose 6-phosphate and 10 mM- NH_4^+ ions; 100% activity = $4.4 \mu\text{mol (mg protein)}^{-1} \text{ min}^{-1}$. AMP, GMP, IMP, CMP and UMP were ineffective (nil activity) at both 1 mM and 10 mM.

Nucleotide	% Activity at:	
	1 mM	10 mM
ATP	100	100
ADP	12	31
GTP	39	98
GDP	4	20
ITP	30	80
IDP	4	ND
CTP	14	63
UTP	5	28
UDP	2	4

ND, Not determined.

for UTP. Nucleotide diphosphates were only partially active as substrates whilst all the nucleotide monophosphates were totally inactive.

The response of PFK to various combinations of adenine nucleotides, to give various simulated energy charge values (cf. Atkinson, 1977), was determined (Fig. 4) as these are probably more important than the effects of the individual nucleotides. The activity of PFK depended upon the total concentration of adenine nucleotides being used. With the lowest concentration, 0.1 mM, which was much below the saturating substrate concentration of the enzyme (for ATP), the activity appeared to be limited by the availability of the substrate. With 1.0 mM-nucleotides, maximum activity was attained at an energy charge of 0.8. With 10 mM-nucleotides, high activity of PFK (greater than 75% maximum) occurred at all energy charge values tested. There was no evidence that ADP, or AMP, either inhibited or stimulated the activity.

Effect of various metabolites

Other likely modulators of PFK activity, besides ATP and NH_4^+ ions, were tested at 10 mM both alone and in the presence of 20 mM- NH_4^+ . With K^+ ions, in the absence of NH_4^+ , activity increased to 139%, but with NH_4^+ present no stimulation was evident. PFK was severely inhibited in the absence of NH_4^+ ions by the tricarboxylic acids, isocitrate, *cis*-aconitate and citrate, having activities of 12, 14 and 15%, respectively, of the control. In the presence of NH_4^+ these inhibitions were alleviated to give activities of 79, 80 and 88%, respectively. Fructose

Fig. 5. Effect of citrate on the activity of purified PFK. Assays contained: Δ , 1 mM-F6P + no NH_4^+ ; $\bullet$, 5 mM-F6P + no NH_4^+ ; $\circ$, 5 mM-F6P + 1 mM- NH_4^+ ; $\blacksquare$, 5 mM-F6P + 8 mM- NH_4^+ ; $\square$, 5 mM-F6P + 20 mM- NH_4^+ .

1,6-bisphosphate was the only other metabolite to result in any inhibition; this, though, was slight: 88% of the control in the absence of NH_4^+ and 92% in its presence. Malate, oxaloacetate, pyruvate, 2-oxoglutarate, glucose 6-phosphate, glucose 1-phosphate, glutamate, aspartate, acetate, acetyl-CoA, CoA and Na^+ had no effect on activity either in the absence or presence of NH_4^+ .

Inhibition by citrate

As citrate plays a key role in lipogenesis in oleaginous yeasts including *Rs. toruloides*, we investigated its effect on PFK in more detail (Fig. 5). In the absence of NH_4^+ , the activity of the enzyme quickly diminished with increasing concentrations of citrate. Even in the presence of 1 mM- NH_4^+ and 5 mM-F6P, citrate was still a strong inhibitor, giving 58% inhibition at 10 mM. When the NH_4^+ concentration was increased to 8 mM, the inhibition by citrate dropped to 28% and at 20 mM- NH_4^+ , PFK was only inhibited by 10%.

From a Lineweaver-Burk plot this inhibition of PFK by citrate was found to be competitive with F6P in the absence of NH_4^+ ions; the K_i values for citrate were calculated as 1.0 mM with zero NH_4^+ ions and 7.2 mM in the presence of 10 mM- NH_4^+ .

The effect of fatty acyl-CoA esters

Both mitochondrial citrate efflux (Evans *et al.*, 1983) and ATP: citrate lyase (Boulton & Ratledge, 1981, 1983b) in oleaginous yeasts have been shown to be extremely sensitive to inhibition by long chain fatty acyl-CoA esters (FACEs). It was therefore thought that PFK might also be susceptible to feedback inhibitory control by these molecules. With FACEs at 10 μM little inhibition was observed: palmitoyl-CoA inhibited activity only by 16%; stearoyl-CoA, oleoyl-CoA and myristoyl-CoA gave 9 to 10% inhibition; lauroyl-CoA gave 5% inhibition and acetyl-CoA and malonyl-CoA both gave none at all. Palmitoyl carnitine and methyl palmitate gave negligible inhibitions (4% and 3%, respectively). At 100 μM , FACEs gave more pronounced inhibitions: palmitoyl-CoA, myristoyl-CoA, stearoyl-CoA, oleoyl-CoA and lauroyl-CoA inhibited activity by 77, 53, 49, 40 and 28%, respectively. However, as the critical micelle concentration of palmitoyl-CoA is about 3 μM (see Boulton & Ratledge, 1983b), the inhibitions induced by the higher concentrations of FACEs are probably not of physiological significance.

DISCUSSION

The growth of *Rs. toruloides* CBS 14 in the presence of NH_4^+ causes the glucose substrate to be channelled preferentially into carbohydrate (mainly glycogen) rather than lipid, even though the cells contain the necessary enzymes for lipid accumulation. By following the uptake of ^{14}C -labelled glucoses, we were able to deduce that there is some impediment to the flow of glucose

Table 4. *Comparison of phosphofructokinase from yeasts*

	<i>S. cerevisiae</i>	<i>Rt. glutinis</i>	<i>Rs. toruloides</i> (this paper)
M_r	835000 (1)	—	700000
K_m (F6P)	0.26 mM (2)	0.55 mM (3) 50 mM (4) > 3 mM (5)	0.55 mM in presence of NH_4^+ 1.25 mM in absence of NH_4^+
K_m (ATP)	0.033 mM (2)	15 mM (3)	0.3–0.4 mM
pH optimum	7.8–8.2 (2)	8.0 (3)	8.0
Inhibition by ATP	Yes, at 0.1–0.5 mM (2, 7, 9)	No (3, 4, 6)	None up to 10 mM
Inhibition by ADP	Yes, at 0.5 mM (8)	Yes, at 100 mM (4)	None up to 10 mM
Inhibition by citrate	Yes (7)	?	Yes
Stimulation by NH_4^+	Yes (8, 9)	?	Yes

References: (1) Kopperschläger *et al.* (1977); (2) Lindell & Stellwagen (1968); (3) Mazon *et al.* (1974); (4) Zeidan (1981); (5) Batrons *et al.* (1982); (6) Doelle & Hollywood (1977); (7) Salas *et al.* (1965); (8) Atzpodien & Bode (1970); (9) Mavis & Stellwagen (1970).

through to acetyl-CoA in these cells; this restriction is removed either when cells are grown with an organic nitrogen source (e.g. glutamate) or when such a nitrogen source is added to washed cell suspensions. As our previous studies have shown that organic nitrogen sources rapidly engender high intracellular concentrations of NH_4^+ , but NH_4^+ itself does not (Evans & Ratledge, 1984*a, b*), we concluded that the most likely explanation for our observations was that NH_4^+ ions were regulating the activity of a key enzyme of glycolysis: the most likely candidate being PFK.

PFK was originally thought to be absent in high lipid-producing species of *Rhodotorula* and *Candida* 107 (Brady & Chambliss, 1967; Höfer *et al.*, 1969, 1971; Whitworth & Ratledge, 1975). It was eventually detected in the red yeast *Rhodotorula glutinis* and *Candida* 107 using the toluene permeabilization method of Serrano *et al.* (1973) and shown to be extremely labile (Mazon *et al.*, 1974; Ratledge & Botham, 1977). The enzyme we have isolated from *Rhodospiridium toruloides* CBS 14 showed a similar lability in extracts prepared from nitrogen-limited cells but was stable when isolated from cells grown with an excess of nitrogen. The former extracts were found to contain a protease which appeared relatively specific for PFK and could thus account for the low activities of PFK which are sometimes reported (e.g. Zeidan, 1981) and for the previous failures to find this enzyme in the *Rhodotorula*. A similar protease has been identified in extracts of *S. cerevisiae* (Diezel *et al.*, 1973), though in this case the subunits of the octameric PFK complex were partially hydrolysed without rendering the enzyme inactive (Kopperschläger *et al.*, 1977). Whether this protease in *Rs. toruloides* fulfils a regulatory role in controlling PFK activity remains unclear at this stage.

The molecular size of PFK from *Rs. toruloides* was 700000, which is slightly less than the 835000 ± 32000 calculated for the enzyme from *S. cerevisiae* (Kopperschläger *et al.*, 1977). Previous estimates of 500000 to 600000 for the size of the enzyme (see Uyeda, 1979) are considered to be of proteolytically modified forms which occur by losses of 24000 M_r fragments from each of the eight subunits. Whilst it is possible that similar proteolysis occurred in the present study, it does not appear to have affected the response of the purified enzyme to various effectors *in vitro*. Although the preparation of PFK had been considerably purified, it had only about 5% of the specific activity of the PFK purified to homogeneity from bakers' yeast (Diezel *et al.*, 1973).

A comparison of the other properties of PFK from *Rs. toruloides* with those of the related *Rt. glutinis* and *Saccharomyces cerevisiae* show some important differences as well as similarities (see Table 4). The most important difference between the PFK from *Rs. toruloides* and that from bakers' yeast was that the former enzyme showed no inhibition by either ATP or ADP up to 10 mM. Both PFKs, however, were activated by NH_4^+ , which significantly increased the affinity of the enzyme for both its substrates. The PFK from bakers' yeast was stimulated 20-fold in the presence of 80 mM- NH_4^+ (Mavis & Stellwagen, 1970) and although, in our case, the stimulation was less (4-fold) it was achieved by significantly less NH_4^+ (10 mM). The enzyme from *Rs. toruloides* was competitively inhibited by citrate and again this has been reported for PFK enzymes

Fig. 6. Role of PFK in lipid accumulation in *Rs. toruloides* CBS 14. + and - indicate action of positive and negative effectors. ATP:citrate lyase (ATP:CL) is the rate-limiting step of fatty acid biosynthesis (Boulton & Ratledge, 1981), therefore citrate will accumulate to a high steady-state concentration before lipid accumulation begins. Citrate will then inhibit PFK, thus channelling glucose into carbohydrate. However, if the intracellular concentration of NH_4^+ is high, as when an organic nitrogen source is used in the medium, this inhibition can be relieved. Under such circumstances, glucose is now converted to lipid. The metabolism of citrate through the citric acid cycle is prevented by the requirement of isocitrate dehydrogenase for AMP (Botham & Ratledge, 1979), which is at a low concentration in lipid-accumulating cells (Boulton & Ratledge, 1983*a*). Citrate, in addition to inhibiting PFK, may also serve to stimulate acetyl-CoA carboxylase (see Botham & Ratledge, 1979).

from other yeasts and micro-organisms (Salas *et al.*, 1965; Rohr & Kubicek, 1981). It is, however, the interrelationship between the inhibition by citrate and the relief of this inhibition by NH_4^+ which we consider to be the key regulatory event which can then explain lipid accumulation in this oleaginous yeast when grown with different nitrogen sources (Evans & Ratledge, 1984*a, b*).

From our previous data (Evans & Ratledge, 1984*a*) it can be calculated that in *Rs. toruloides* CBS 14, the intracellular citrate concentrations increased from approx. 2 mM to 8 mM, between 20 and 40 h growth, in both glutamate- and urea-grown cells (values calculated assuming an intracellular water space of 2 μl per mg dry weight; Knowles, 1977). It is difficult to ascertain the significance of these concentrations because of compartmentation within the cell but the excretion of citrate into the medium before lipid starts to accumulate suggests that the cytosolic pool of citrate has reached its maximum concentration (Evans & Ratledge, 1984*b*; see also Boulton & Ratledge, 1983*a*). The actual cytosolic concentration of citrate in the vicinity of PFK could be much greater than 2 to 8 mM because the intracellular aqueous volume will be decreased by the expanding size of the lipid globules which can account for half the cell volume (Evans & Ratledge, 1984*a*).

If we assume intracellular citrate concentrations could be as high as 10 mM, then PFK activity would be completely inhibited as the actual substrate (F6P) concentration will probably be very low throughout growth (the concentration of glucose 6-phosphate, with which F6P will be in equilibrium, was usually about 10% of the citrate concentration in these cells – Evans & Ratledge, 1984a). However, the intracellular NH_4^+ concentration reaches 15 mM after 30 h growth on glutamate (Evans & Ratledge, 1983a, b) which would ensure almost maximum PFK activity even in the presence of this amount of citrate (see Fig. 6). From the work of Boulton & Ratledge (1983a) and Botham & Ratledge (1979) a value of about 1 mM can be calculated for the concentrations of ATP within oleaginous yeasts. This would be a sufficient concentration, if also present in *Rs. toruloides*, not to be limiting PFK activity during lipogenesis.

This type of regulation of PFK *in vivo* by NH_4^+ ions is similar to that reported in other systems (Abrahams & Younathan, 1971; Bloxham & Lardy, 1973; Lloyd *et al.*, 1983) and for the citric acid-producing aspergilli (Habison *et al.*, 1983; Rohr & Kubicek, 1981) where very high concentrations of intracellular NH_4^+ ions (between 25 mM and 15 mM) prevail throughout growth providing constant alleviation of citrate inhibition. We conclude, therefore, that when NH_4^+ is used as nitrogen source with *Rs. toruloides* and the intracellular concentration of NH_4^+ is low (Evans & Ratledge, 1984a), PFK is readily inhibited by citrate. Clearly, some glucose is metabolized through PFK in order to maintain the tricarboxylic acid cycle and the citric acid pool. Even when cells have exhausted the supply of nitrogen and lipid accumulation may be expected to occur, PFK continues to be inhibited. Consequently incoming glucose is now diverted into the only other storage material possible – carbohydrate. Such cells could be described as exhibiting the properties of the classical Pasteur effect, whereby glycolysis is restricted and carbohydrate reserves build up (Sols *et al.*, 1971).

When cells are grown with an organic nitrogen source, such as glutamate or urea, the intracellular NH_4^+ concentration is high during the first 40 h growth (Evans & Ratledge, 1984a), PFK is fully active and lipid accumulation can then commence. However, although our present observations explain how lipid accumulation takes place during the first 40 h they do not provide an explanation for its continued synthesis after this time when it would be expected that the ameliorating effect of NH_4^+ on citrate inhibition of PFK would disappear after exhaustion of the nitrogen source. The findings of Mavis & Sellwagen (1970), however, show that the PFK from *S. cerevisiae* forms two complexes with NH_4^+ ; if the enzyme from *Rs. toruloides* were to form similar complexes these may persist after the depletion of the intracellular NH_4^+ pool thus ensuring continued insensitivity to citrate inhibition. Whatever the explanation, the metabolic machinery of the cell, which includes PFK, becomes 'locked-in' during the first 40 h growth so that subsequent changes in concentrations of key metabolites do not then deflect the cell from subsequently accumulating lipid.

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