Phosphoketolase in *Rhodotorula graminis* and Other Yeasts

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Phosphoketolase(s), catalysing the cleavage of xylulose 5-phosphate and fructose 6-phosphate, were found in *Rhodotorula graminis*, *R. glutinis*, *Candida* 107 and *C. tropicalis*. Activity towards the latter substrate only was found in *C. humicola*. Neither activity was found in *Saccharomyces carlsbergensis*. The enzyme(s) were isolated and purified eightfold from *R. graminis*. Inorganic phosphate, thiamin pyrophosphate and Mg^{2+} were required for activity. The enzyme had a K_m of 1.25 mM for xylulose 5-phosphate and was markedly sensitive to NADH, NADPH, ATP and acetyl-CoA and less sensitive to phosphoenolpyruvate, citrate and dodecanoyl-CoA. The activity of phosphoketolase was halved in the presence of an active transketolase which competes with it for the same substrate. The contribution of phosphoketolase to glucose catabolism may be only slight.

INTRODUCTION

The apparent absence of phosphofructokinase in species of *Rhodotorula* (Brady & Chambliss, 1967; Höfer, 1968) led to a proposal that phosphoketolase, catalysing

xylulose 5-phosphate $\longrightarrow$ glyceraldehyde 3-phosphate + C_2 unit
or
fructose 6-phosphate $\longrightarrow$ erythrose 4-phosphate + C_2 unit

was present as an adjunct to the pentose cycle (Höfer *et al.*, 1969, 1971; Höfer, Betz & Becker, 1970). Such a proposal was needed as the pentose cycle produces only one C_2 unit from each molecule of glucose. The presence of phosphoketolase was never verified, though glyoxylate reductase, catalysing

was found (Höfer *et al.*, 1970), and this was said to confirm the existence of phosphoketolase and also that the C_2 product of its reaction was glycollate. However, the nature of the C_2 units is probably 2-(α,β -dihydroxyethyl)thiamin pyrophosphate (enzyme-bound) which can break down to a number of products including glycollate or acetyl phosphate depending on the assay conditions (Horecker, 1962; Goldberg & Racker, 1962; Schröter & Holzer, 1963).

As at this stage we had also failed to find phosphofructokinase in several oleaginous yeasts including *Rhodotorula* spp. and *Candida* 107 (Whitworth & Ratledge, 1975*a, b*), we subsequently searched for, and found, phosphoketolase in these yeasts. We now present some of the properties of this enzyme which has hitherto only been recognized in the heterofermentative lactobacilli and the bifidobacteria (Doelle, 1969; Sgorbati, Lenaz & Casalicchio, 1976).

The enzyme, however, probably does not have the metabolic importance previously

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attached to it as the conventional glycolytic pathway has now been established in *R. glutinis*, *R. graminis* and *Candida* 107 by the finding of phosphofructokinase (Mazón, Gancedo & Gancedo, 1974; Ratledge & Botham, 1977).

METHODS

Growth of yeasts and preparation of cell-free extracts. *Candida* 107 Ruinen & Deinema 1964, *Candida humicola* NCYC461, *Candida tropicalis* NCYC3, *Rhodotorula graminis* NCYC502, *Rhodotorula glutinis* (syn. *gracilis*) NCYC59 and *Saccharomyces carlsbergensis* NCYC30 were grown on a glucose/salts medium at pH 5.5 as described by Ratledge & Botham (1977). The yeasts, usually grown in 100 ml medium in 250 ml conical flasks for 48 h, were harvested, washed and disrupted as previously described (Whitworth & Ratledge, 1975a). Extracts were centrifuged at 45000 g for 30 min and the supernatant fluids were used in assays. Protein was measured by the biuret method (Gornall, Bardawill & David, 1949).

Enzyme assays

Phosphoketolase(s) [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)]. Two discontinuous methods were used, the first being suitable for assaying hexose phosphoketolase, the second for pentose phosphoketolase. Unless stated otherwise all results refer to the enzyme assayed by the latter procedure.

(i) Assay of acetyl phosphate as ferric acetyl hydroxamate (based on the method of Racker, 1962; and method B of Goldberg, Fessenden & Racker, 1966). The reaction mixture contained, in 1 ml: cell extract, about 0.1 ml; histidine/HCl buffer, pH 6.0, 50 mM; KH_2PO_4 /KOH buffer, pH 6.0, 20 mM; MgCl_2 , 1 mM; dithiothreitol, 2 mM; thiamin pyrophosphate (TPP), 0.3 mM; and, to initiate the reaction, fructose 6-phosphate, 20 mM. After incubating at 30 °C for 15 min, 0.5 ml 2 M-hydroxylamine hydrochloride, pH 5.4, was added and, 10 min later, 1.5 ml 20% (w/v) FeCl_3 in 0.1 M-HCl. After centrifuging, E_{440} was measured immediately to give the amount of acetyl phosphate formed.

(ii) Assay of glyceraldehyde 3-phosphate by its enzymic conversion to fructose 1,6-bisphosphate (Goldberg *et al.*, 1966; method A). The reaction mixture contained, in 150 μl : cell extract, about 25 μl ; KH_2PO_4 /KOH buffer, pH 6.0, 33 mM; dithiothreitol, 3.3 mM; MgCl_2 , 3.3 mM; TPP, 0.7 mM; α -glycerophosphate dehydrogenase, 0.2 unit; triosephosphate isomerase, 1.6 units; aldolase, 0.1 unit; and, to initiate the reaction, xylulose 5-phosphate, 1.7 mM. After 5 min at 30 °C, the tubes were held in boiling water for 1 min. When cool, the precipitate was removed by centrifuging and the amount of fructose 1,6-bisphosphate formed from the glyceraldehyde 3-phosphate was measured in 50 μl of the supernatant solution by adding it to 50 mM-triethanolamine/HCl buffer, pH 7.5, plus glyceraldehyde-3-phosphate isomerase, α -glycerophosphate dehydrogenase and aldolase (0.1 unit of each) and 0.1 mM-NADH, all in a final volume of 1 ml. Measurement of ΔE_{340} gave the amount of glyceraldehyde 3-phosphate formed over the initial incubation period. As a control, the assay was conducted in the absence of xylulose 5-phosphate.

Acetate kinase [EC 2.7.2.1; ATP:acetate phosphotransferase] was assayed in the forward direction by the method of Rose (1955) and in the reverse direction in a similar assay system but using 15 mM-acetyl phosphate as substrate. Both assays were discontinuous and the amount of acetyl phosphate present was determined after 30 min incubation.

Transketolase [EC 2.2.1.1; sedoheptulose-7-phosphate:D-glyceraldehyde-3-phosphate glycolaldehyde-transferase] was assayed by the method of Kiely, Tan & Wood (1969) by coupling with α -glyceraldehyde dehydrogenase/triosephosphate isomerase and following oxidation of NADH at 340 nm.

Purification of phosphoketolase. *Rhodotorula graminis* was cultured in 1 l medium in a 2 l conical flask with continuous shaking for 4 days. The cell-free extract was prepared as above and centrifuged at 100000 g for 1 h. To the supernatant solution, streptomycin sulphate was added at 1.5 mg (mg protein)⁻¹. After removing precipitated material, the supernatant was fractionated with ammonium sulphate. The 50 to 60% saturated fraction was dialysed against 50 mM-phosphate buffer, pH 7.5, and then applied to a column (10 × 30 mm) of hydroxylapatite which was eluted with a gradient of phosphate buffer from 50 to 250 mM. Active fractions were combined and used in further experiments.

Biochemicals were obtained from Sigma.

RESULTS AND DISCUSSION

Of the various supernatant solutions of disrupted yeasts which were examined for phosphoketolase activity (Table 1), the most promising source for further study appeared

Table 1. Activity of phosphoketolase in extracts from various yeasts

Phosphoketolase activity was assayed with the coupled enzyme assay method with xylulose 5-phosphate (Xu 5-P) as substrate. With fructose 6-phosphate (F 6-P) as substrate, the acetyl hydroxamate method was used which consistently gave no activity when the substrate was omitted. Activities are expressed as nmol substrate converted min^{-1} (mg protein^{-1}).

Yeast	Xu 5-P substrate		F 6-P substrate
	Complete assay	Assay—substrate	Complete assay
<i>S. carlsbergensis</i>	4.7	7.0	0.5
<i>C. humicola</i>	18.9	20.8	10.0
<i>Candida</i> 107	3.1	0	1.7
<i>C. tropicalis</i>	8.3	2.5	1.4
<i>R. graminis</i>	4.4	1.5	8.7
<i>R. glutinis</i>	5.1	1.0	1.9

to be that from *R. graminis*. The enzyme was not detected in *S. carlsbergensis*. *Candida humicola* gave a high specific activity only when fructose 6-phosphate was the substrate.

Since the assays with fructose 6-phosphate as substrate were conducted with crude extracts, enzymes producing or degrading acetyl phosphate might have interfered. Acetate kinase was assayed in both directions but the level of its activity was very much less than that due to phosphoketolase. Extracts were also assayed for glyoxylate reductase activity (see Höfer *et al.*, 1969) in both directions by the method of Zelitch & Gotto (1962) but no significant levels were found.

Purification and properties of phosphoketolase from *R. graminis*

Phosphoketolase from *R. graminis* was purified twofold after ammonium sulphate precipitation and a further fourfold by chromatography through hydroxylapatite with an overall recovery of activity of 12 %. (Phosphoketolase from *Candida* 107 was not successfully purified using this procedure.) Neither calcium phosphate gel, alumina C_γ nor Sephadex G-150 was suitable as a final purification step. Fractions eluted from the hydroxylapatite column, which showed a peak of activity with fructose 6-phosphate as substrate, preceded those showing optimum activity with xylulose 5-phosphate. Therefore, although different assay procedures were used with each substrate, hexose and pentose phosphoketolase activities might not belong to the same enzyme.

The purified preparation lost 50 % of its activity in 24 h at 4 °C. Inorganic phosphate was essential to maintain stability; when phosphate buffer was replaced by equimolar Tris/maleate buffer at the same pH, complete loss of activity occurred within 30 min. TPP was also essential for activity. Activity diminished upon dialysis but could be restored by adding Mg^{2+} . The enzyme exhibited its maximum activity at pH 6.0; at pH 5.5 and pH 7.0 the activity was less than 40 % of the maximum. The optimum temperature for the assay was 30 °C; at 27.5 °C the activity was 70 % of the maximum and at 32.5 °C was 30 %. Under the optimum conditions of assay, the enzyme had a K_m of 1.25 mM for xylulose 5-phosphate calculated from a double reciprocal plot in the usual manner.

The enzyme was inhibited by NADH, NADPH, phosphoenolpyruvate, citrate, ATP, acetyl-CoA and dodecanoyl-CoA (Table 2). None of these metabolites affected the activity of the coupling enzyme, i.e. if added after the reaction was stopped. (NADH and NADPH, though they could not be checked in this way as the second part of the assay requires NAD(P)H to be added at 0.1 mM, would clearly not interfere with the assay procedure.) The enzyme was particularly sensitive to NADH, NADPH, ATP and acetyl-CoA. The inhibition by reduced nicotinamide coenzymes was not relieved by adding higher concentrations of the oxidized coenzymes.

Table 2. *Effect of various metabolites on phosphoketolase activity*

Phosphoketolase activity was assayed by estimation of glyceraldehyde 3-phosphate using xylulose 5-phosphate as substrate; activities are expressed as $\text{nmol min}^{-1} (\text{mg protein})^{-1}$. Neither citrate, phosphoenolpyruvate, ATP (at 2 mM) nor acetyl-CoA (at 0.2 mM) affected the estimation of glyceraldehyde 3-phosphate. NADH and NADPH could not be so tested as NAD(P)H (at 0.1 mM) is already added to the coupled enzyme assay system.

Effector	Concn (mM)	Phosphoketolase activity
None		108
NADH	0.2	0
	0.05	22
NADH+NAD	0.1+0.5	22
NADPH	0.2	0
	0.05	38
NADPH+NADP	0.1+0.5	5
Phosphoenolpyruvate	10	27
	2	60
Citrate	10	27
	2	40
ATP	0.1	0
	0.01	51
Acetyl-CoA	0.1	0
	0.05	27
Dodecanoyl-CoA	0.5	11

Phosphoketolase and transketolase activities

Transketolase converts xylulose 5-phosphate with either ribose 5-phosphate or erythrose 4-phosphate into glyceraldehyde 3-phosphate and either sedoheptulose 7-phosphate or fructose 6-phosphate. Its assay is similar to that for phosphoketolase as it uses the same substrate and relies on the formation and subsequent enzymic dehydrogenation of glyceraldehyde 3-phosphate. When enzyme preparations were assayed for transketolase, full activity [$485 \text{ nmol product formed min}^{-1} (\text{mg protein})^{-1}$] was found with xylulose 5-phosphate and ribose 5-phosphate together. Without the former substrate the activity dropped by 67% and without the latter no activity occurred. When the phosphoketolase assay was carried out by following the formation of acetyl phosphate (i.e. in the assay usually used with fructose 6-phosphate as substrate) with both xylulose 5-phosphate and ribose 5-phosphate present, its activity was halved due to the competition of transketolase for the substrate. Thus, both the pentose phosphate cycle and the phosphoketolase pathway compete for the same substrate, xylulose 5-phosphate.

The contribution which phosphoketolase makes to glucose catabolism may be only slight. Its low activity, sensitivity to intracellular metabolites and its competition with transketolase for its substrate are not the hallmarks of a major controlling enzyme. Why phosphoketolase should be present at all is a puzzle. As its purpose is to divert carbon from the pentose cycle into the tricarboxylic acid cycle it could only play a major role if the Embden-Meyerhof pathway was absent or functioning at a low level and this possibility has been eliminated by the recent discoveries of phosphofructokinase in *R. glutinis*, *R. graminis* and *Candida* 107 (Mazón *et al.*, 1974; Ratledge & Botham, 1977). Perhaps as we understand more of the control and regulation of glucose metabolism in these yeasts the significance of phosphoketolase will become apparent. Speculation at this stage is therefore unwarranted.

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