Characteristics of Fructose-1,6-bisphosphatase from the Methanol-utilizing Yeast *Hansenula polymorpha*

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Fructose-1,6-bisphosphatase (EC 3.1.3.11) has been partially purified from methanol- and ethanol-grown *Hansenula polymorpha*. The enzyme was specific for fructose 1,6-bisphosphate as a substrate and did not react with sedoheptulose 1,7-bisphosphate. The enzyme purified from ethanol-grown cells resembled that from methanol-grown cells in all properties investigated, namely, temperature stability, substrate affinity, pH-dependent inhibition by AMP, and pH-dependent stimulation of activity by EDTA. It is concluded that, although the metabolic role of fructose-1,6-bisphosphatase during growth on methanol and ethanol is completely different, growth on these substrates probably involves the same enzyme.

INTRODUCTION

As a result of enzymic, mutant and whole-cell studies, it has been established that growth of yeasts on methanol proceeds via the xylulose monophosphate (Xu5P) pathway of formaldehyde fixation (van Dijken *et al.*, 1978; Babel & Loffhagen, 1979; Kato *et al.*, 1979; O'Connor & Quayle, 1979; Waite & Quayle, 1980; Lindley *et al.*, 1980). The key reaction of this cycle (Fig. 1) is a transketolase type of condensation between xylulose 5-phosphate and formaldehyde, which results in the formation of C₃ compounds (glyceraldehyde 3-phosphate and dihydroxyacetone). The rearrangement of these reaction products to xylulose 5-phosphate proceeds via fructose 1,6-bisphosphate and fructose 6-phosphate. Therefore fructose-1,6-bisphosphatase (FBPase), which is present at enhanced levels during growth of yeasts on methanol (van Dijken *et al.*, 1978), fulfils an essential role in methanol metabolism since the synthesis of all cell constituents proceeds via this reaction. In contrast when growth occurs on C₂, C₃ and C₄ compounds the enzyme has a role in gluconeogenesis only. The Xu5P pathway of formaldehyde fixation is similar to the Calvin cycle. Thus during autotrophic fixation of CO₂, FBPase has a similarly important role in the regeneration of the C-5-acceptor molecule from the reaction products of the condensation, i.e. phosphoglycerate (Springgate & Stathow, 1972; Joint *et al.*, 1972; Abdelal & Schlegel, 1974). It was, therefore, of interest to study the properties of the FBPase from a methylotrophic yeast, particularly since in some bacteria the FBPase functioning during autotrophic growth is reported to be different from that operating during growth on substrates that require gluconeogenesis (Amachi & Bowien, 1979). The results reported in this communication indicate that the FBPase which is present in the yeast *Hansenula polymorpha* during growth on methanol is very similar in its properties to that present in ethanol-grown cells and resembles the enzyme commonly encountered in non-methylotrophic yeasts.

METHODS

Organism and growth. *Hansenula polymorpha* (CBS 4732) was grown in shake flasks on a mineral medium containing 0.5% (v/v) methanol or ethanol as described previously (van Dijken *et al.*, 1978).

Preparation of extracts. Cell-free extracts were prepared by ultrasonication (MSE model 150W) at 4 °C for 5 × 1 min in 4 vol 100 mM-KH₂PO₄/NaOH buffer pH 7.5 containing 5 mM-MgCl₂, in the presence of glass beads.

Preparations were centrifuged at 18000 *g* for 20 min at 4 °C and the supernatants used as the cell-free extract.

Partial purification of FBPase. Crude cell-free extracts were brought to pH 5.5 by the slow addition of 0.3% (v/v) acetic acid; centrifuged (18000 *g*, 20 min, 4 °C) and the supernatant brought to pH 7.5 by the addition of 1 M-KOH. The supernatant was taken to 50% saturation with (NH₄)₂SO₄ by the slow addition of solid (NH₄)₂SO₄ at 4 °C. The pH was adjusted to 7.5 with NH₄OH and desalted with a Sephadex G-25 column (40 × 2.5 cm) equilibrated with 20 mM-KH₂PO₄/NaOH buffer pH 7.5. The pooled active fractions were applied directly to a column (7.5 × 2 cm) of hydroxylapatite (Bio-rad) which had been equilibrated with the same buffer. After the sample had been washed on to the column with 80 ml of this buffer it was eluted with a linear phosphate gradient (20–500 mM made up in 500 ml of the same buffer). Active fractions were pooled and concentrated using an Amicon ultrafiltration unit fitted with a PM10 Diaflo membrane (Amicon) to a concentration of 10 units ml⁻¹. The preparation was stored at –20 °C. When the discontinuous assay, measuring the release of inorganic phosphate, was used, the Amicon filter preparation was added to the top of a Sephadex G-25 column (20 × 1.5 cm) equilibrated in and eluted with 50 mM-Tris/HCl buffer pH 7.5.

Enzyme assays. All enzyme assays were carried out at 37 °C in a Pye-Unicam SP1800 double-beam recording spectrophotometer.

Unless stated otherwise FBPase (EC 3.1.3.11) was assayed by determining the rate of fructose 6-phosphate formation. The reaction mixture contained, in a final volume 1 ml: Tris/HCl buffer pH 8.5, 100 mM; MgCl₂, 5 mM; EDTA, 1 mM; NADP, 0.4 mM; glucose-6-phosphate isomerase (EC 5.3.1.9), 1 unit; glucose-6-phosphate dehydrogenase (EC 1.1.1.49), 1 unit. The reaction was started by the addition of fructose 1,6-bisphosphate, 2 mM. When the effect of various inhibitors on the enzyme was investigated the compounds were pre-incubated for 5 min at 37 °C in the assay mixture and the reaction started by the addition of fructose 1,6-bisphosphate.

For the determination of the substrate specificity of the enzyme the rate of inorganic phosphate release was measured. The reaction mixture was the same as that described for the spectrophotometric assay without the linkage enzymes. The inorganic phosphate released was determined by the method of Ames (1966).

Fructose-bisphosphate aldolase (EC 4.1.2.13) was assayed according to van Dijken *et al.* (1978).

Specific activities are expressed as units (mg protein)⁻¹. One unit is defined as 1 μmol substrate consumed or product formed min⁻¹.

Analytical methods and chemicals. Protein was estimated by the Lowry method using bovine serum albumin as standard. Biochemicals and enzymes were supplied by Boehringer.

RESULTS AND DISCUSSION

FBPase was purified 13-fold from methanol-grown *H. polymorpha* with a recovery of 40%. The final preparation had a specific activity of 10.3 units (mg protein)⁻¹ and contained some FBP aldolase activity [0.15 units (mg protein)⁻¹]. However, under the conditions of the FBPase assay the aldolase activity was zero. This enabled an estimation of the *K_m* of the FBPase for fructose 1,6-bisphosphate to be obtained. Published procedures for the purification of this enzyme from *Candida utilis* (Rosen *et al.*, 1965) such as heat treatment and precipitation with (NH₄)₂SO₄ at high saturation values could not be used since they resulted in an irreversible loss of enzyme activity. Attempts to purify the enzyme by gel filtration gave inconsistent results, probably due to the dissociation of the enzyme into subunits.

The enzyme preparation was rather unstable, having a half life of 1 week at –20 °C, 1 h at 30 °C, and 5 min at 37 °C. This instability is not uncommon in FBPases from yeast. The stability of the enzyme appears to relate to the species of yeast from which the enzyme has been purified. The enzyme from *Saccharomyces cerevisiae* was stable at 4 °C, whereas in extracts of *Pichia vini* and *Rhodotorula gracilis* the half life of the enzyme was 1 and 3 h, respectively, when stored at the same temperature (Gancedo & Gancedo, 1971). The optimum pH for activity was 8.5 when assayed with the standard procedure. The enzyme required magnesium for maximal activity. Manganese could substitute for magnesium with 50% efficiency. The chelating agent EDTA markedly stimulated the enzyme activity at neutral pH values but almost full enzyme activity was observed at high (alkaline) pH values in the absence of EDTA. This stimulating effect of EDTA was more pronounced when the enzyme was in Tris/HCl buffer rather than in glycine/NaOH buffer, and may be due to the possible chelating effect of glycine in the latter buffer. When the enzyme activity was measured in the presence of a range of Mg²⁺ and EDTA concentrations the ratio of Mg²⁺ to EDTA in the reaction mixture was a more important parameter than the individual concentrations of Mg²⁺ or EDTA alone.

The enzyme was specific for fructose 1,6-bisphosphate as a substrate. Sedoheptulose 1,7-

Fig. 1. The central role of FBPase in the formation of cell constituents via the xylulose phosphate pathway of formaldehyde fixation. Xu5P, xylulose 5-phosphate; DHA, dihydroxyacetone; DHAP, dihydroxyacetone phosphate; GAP, glyceraldehyde 3-phosphate; FBP, fructose 1,6-bisphosphate; F6P, fructose 6-phosphate; PGA, phosphoglycerate.

bisphosphate, ribose 5-phosphate, glucose 6-phosphate or fructose 1-phosphate were not substrates. The apparent K_m for fructose 1,6-bisphosphate was 2.9×10^{-5} M.

A variety of compounds were tested as possible modulators of enzyme activity at concentrations between 0.1 and 2 mM. Of those tested (ATP, ADP, AMP, GSH, CoA, xylulose 5-phosphate, ribulose 5-phosphate, ribose 5-phosphate and dihydroxyacetone) only AMP had a significant effect on the enzyme. The inhibition exerted by AMP was dependent on the pH of the assay and decreased as the pH increased.

FBPase was also purified 45-fold from ethanol-grown cells of *H. polymorpha* with a recovery of 80%. The final preparation had a specific activity of 9.6 units (mg protein)⁻¹. The properties of this preparation were essentially similar to that obtained from methanol-grown cells. It was labile, specific for fructose 1,6-bisphosphate as a substrate, with a K_m of 2.2×10^{-5} M, stimulated by EDTA at neutral pH values and inhibited by AMP.

Thus the FBPase from methanol- and ethanol-grown *H. polymorpha* is very similar, if not identical, and resembles the enzyme from *C. utilis* (Rosen *et al.*, 1965). Unlike the situation in *Nocardia opaca* 1b (Amachi & Bowien, 1979) and *Thiobacillus* A2 (Wood *et al.*, 1977), in which different isoenzymes of FBPase are synthesized depending on the substrate for growth, in *H. polymorpha* it is probable that only one FBPase is synthesized irrespective of the growth substrate. Moreover, unlike FBPase from mammalian liver (Bonsignore *et al.*, 1963), *Rhodopseudomonas palustris* (Springgate & Stathow, 1972) and *Hydrogenomonas eutropha* (Abdelal & Schlegel, 1974), but similar to the enzyme from *C. utilis* (Rosen *et al.*, 1965) and *Escherichia coli* (Fraenkel *et al.*, 1966), the *H. polymorpha* FBPase is specific for fructose 1,6-bisphosphate and does not react with sedoheptulose 1,7-bisphosphate. Since sedoheptulose bisphosphatase activity was also absent in crude cell-free extracts of methanol-grown *H. polymorpha*, it must be concluded that the enzyme is functioning as shown in Fig. 1 and does not play a role in a rearrangement reaction in which it functions as a sedoheptulose bisphosphatase in conjunction with a transketolase (see Zatman, 1981).

Apart from its substrate specificity, the FBPase purified from *H. polymorpha* resembles the FBPase from other micro-organisms (Springgate & Stathow, 1972; Abdelal & Schlegel 1974). Thus, it requires cations for activity (Mg^{2+} or Mn^{2+}), exhibits a pH optimum in the alkaline range, and has a similar K_m for fructose bisphosphate. Furthermore, like most FBPases it is stimulated by chelating agents (EDTA) particularly at neutral pH values (Rosen *et al.*, 1965; Mikkada & Bell, 1977; Van Tol *et al.*, 1972; Nimmo & Tipton, 1975; Pontremoli *et al.*, 1979).

An important aspect of the regulatory properties of FBPase is the significance of the *in vitro* regulation and its activity by AMP. FBPases purified from mammalian sources and heterotrophic bacteria are inhibited by AMP (Taketa & Pogell, 1965; Opheim & Bernlohr, 1975) whereas the FBPases from plant chloroplasts (Buchanan *et al.*, 1971) and photolithoautotrophic and chemolithoautotrophic bacteria are insensitive to AMP but inhibited by ATP (Joint *et al.*, 1972; Springgate & Stathow, 1972; Abdelal & Schlegel, 1974). The physiological rationale for AMP inhibition of FBPases in animals and heterotrophic bacteria grown on C₂, C₃ and C₄ compounds is in the control of gluconeogenesis, since if FBPase is inhibited by AMP and not ATP the cell would be protected against a wasteful dephosphorylation of fructose 1,6-bisphosphate during active glycolysis (see review by Pontremoli & Horecker, 1971). However, for two reasons, it is doubtful whether the *in vitro* inhibition of FBPase by AMP plays any regulatory role *in vivo* during growth of *H. polymorpha* on methanol. First, the major function of the enzyme during growth on this substrate is the regeneration of the assimilation products to form xylulose 5-phosphate for formaldehyde fixation (Fig. 1), and the formation of fructose 1,6-bisphosphate itself would be decreased in the cell under conditions of low energy charge, due to a decreased phosphorylation of dihydroxyacetone by triokinase. Secondly, any regulatory role for the FBPase during growth on methanol would be difficult to explain since the FBPase purified from ethanol-grown *H. polymorpha* resembled that purified from methanol-grown cells in all the properties investigated, even though during growth on ethanol the enzyme has a role in gluconeogenesis only.

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