The Interrelation between Transketolase and Dihydroxyacetone Synthase Activities in the Methylotrophic Yeast *Candida boidinii*

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Crude extracts of *Candida boidinii* grown on glucose, xylose or ethanol gave single peaks of classical transketolase activity following chromatography on columns of hydroxylapatite; the enzyme was heat-stable and showed no appreciable activity with formaldehyde as acceptor in place of ribose 5-phosphate. Extracts of methanol-grown cells showed two peaks of transketolase activity following chromatography on both hydroxylapatite and DEAE-cellulose. One peak was identified with that found for the cells grown on substrates other than methanol; the other peak showed dihydroxyacetone synthase activity in addition to transketolase activity. Both activities in the latter peak were very unstable and have been ascribed to one enzyme on the basis of identical rates of denaturation at all temperatures tested between 0 and 40 °C. It is suggested that this enzyme is a special transketolase synthesized only during methylotrophic growth of the yeast and, in contrast to classical transketolase, is capable of using equally well either formaldehyde or ribose 5-phosphate as glycolaldehyde acceptor. A method based on heat treatment has been suggested for the simultaneous assay of both transketolases present in crude extracts of a methylotrophically grown yeast.

INTRODUCTION

A key step in the pentose phosphate cycle of formaldehyde fixation which has been found to operate in growth of methylotrophic yeasts on methanol is the transfer of a glycolaldehyde fragment (most probably donated by xylulose 5-phosphate) to formaldehyde, with the formation of dihydroxyacetone (DHA) and glyceraldehyde 3-phosphate, catalysed by a special type of transketolase (Kato *et al.*, 1979; Waite & Quayle, 1980; O'Connor & Quayle, 1980). Unlike the classical transketolase (EC 2.2.1.1) found in a variety of tissues, this special transketolase (DHA synthase) from *Candida boidinii* or *Hansenula polymorpha* appeared to be unstable and consequently difficult to purify (O'Connor & Quayle, 1980). It has been shown that DHA synthase activity is only present in methanol-grown cells (Kato *et al.*, 1979; Waite & Quayle, 1980; O'Connor & Quayle, 1980) and this activity has been separated from a peak of classical transketolase activity by ion-exchange chromatography of extracts of methanol-grown *C. boidinii* (O'Connor & Quayle, 1980). By contrast, classical transketolase activity is present in extracts of *C. boidinii* and *H. polymorpha* at relatively high levels during growth on many substrates, including methanol (O'Connor & Quayle, 1980). It has now been discovered that DHA synthase itself exhibits classical transketolase activity; the evidence for this finding and interrelationships between these different enzyme activities during growth of *C. boidinii* on several substrates are described in the present paper. Throughout the paper the term 'classical transketolase' will be used to describe an enzyme which catalyses glycolaldehyde transfer between xylulose 5-phosphate as donor and ribose 5-phosphate as acceptor.

METHODS

Abbreviations. DHA, dihydroxyacetone; DHAP, dihydroxyacetone phosphate; GAP, glyceraldehyde 3-phosphate; RiMP, ribose 5-phosphate; TPP, thiamin pyrophosphate; XuMP, xylulose 5-phosphate.

Organism and growth conditions. *Candida boidinii* CBS 5777 was grown in shake flasks at 30 °C in the mineral salts medium of van Dijken *et al.* (1976). Filter-sterilized methanol (0.5%, w/v) or ethanol (0.2%, w/v) and autoclaved glucose or xylose (0.2%, w/v) were used as carbon and energy sources. For all studies, cultures were harvested in late-exponential phase by centrifugation at 6000 g for 20 min.

Preparation of extracts. Cell-free extracts were prepared by ultrasonication (MSE model 150 W) at 0 °C for 6 × 1 min at 1 min intervals in 3 vol. 10 mM-KH₂PO₄/NaOH buffer pH 7.1 containing 0.005% (w/v) TPP (for purification by hydroxylapatite chromatography) or 20 mM-glycylglycine buffer pH 7.6 containing 5 mM-MgCl₂ and 0.005% (w/v) TPP (for purification by DEAE-cellulose chromatography). Supernatants obtained following centrifugation at 38 000 g for 20 min were used for all enzyme studies.

Enzyme assays. All assays were carried out in 1 ml total volume at 30 °C using a Pye Unicam SP1800 double-beam recording spectrophotometer.

Transketolase (EC 2.2.1.1) was assayed by a modification of the method of de la Haba *et al.* (1955) using an equilibrium mixture of pentose phosphates generated by the addition of RiMP (initial concentration 5 mM) in the presence of RiMP isomerase (EC 5.3.1.6; 1.5 units), ribulose-5-phosphate 3-epimerase (EC 5.1.3.1; 1.5 units) and excess of a commercial mixture of triosephosphate isomerase (EC 5.3.1.1) and glycerol-3-phosphate dehydrogenase (EC 1.1.1.8) (Boehringer).

DHA synthase activity was assayed using a test system containing: glycylglycine buffer pH 7.6, 50 mM; MgCl₂, 2 mM; NADH, 0.15 mM; TPP, 0.005% (w/v); ATP, 1 mM; triosephosphate isomerase, 6 units; glycerol-3-phosphate dehydrogenase, 0.4 unit; glycerol kinase (EC 2.7.1.30; from *Candida mycoderma*), 2 units. A 'blank' system was also set up containing glycylglycine buffer, MgCl₂ and NADH at the same concentrations. A sample of cell-free extract was added to both blank and test cuvettes, which were then incubated in the spectrophotometer for 3 min. During this time no significant change in absorbance occurred as NADH oxidase activity was the same in each cuvette. XuMP then was added to the test cuvette to a final concentration of 2.5 mM, and an equal volume of distilled water was added to the blank. XuMP-dependent NADH oxidation (due to classical transketolase activity, resulting from the presence of pentose phosphate-interconverting activities and also the possible contamination of the commercial XuMP with RiMP) was monitored for 3 to 4 min to allow a steady rate to become established. Formaldehyde was then added to both cuvettes to a final concentration of 1 mM. The observed increase in the rate of NADH oxidation was taken to be the result of formaldehyde- and XuMP-dependent formation of triose phosphates. Any contribution of formaldehyde reductase activity was compensated for by a similar rate occurring in the blank cuvette and being automatically subtracted from that occurring in the test cuvette. In crude extracts a discontinuous method, based on measurement of the XuMP-dependent rate of removal of formaldehyde (Waites & Quayle, 1980), was also used where indicated.

The unit of activity was taken to be that amount of enzyme which catalysed the formation of 1 µmol DHA min⁻¹ under the conditions of assay. In the continuous spectrophotometric assay, calculation of the number of units entailed halving the rate of NADH oxidation observed since two triose phosphate equivalents were produced and estimated for each equivalent of DHA initially generated (see Results).

Partial purification of transketolase and DHA synthase. Method 1: Hydroxylapatite chromatography. Crude cell-free extracts (10 to 25 ml) containing 100 to 200 mg protein were applied directly to a column (10 × 2 cm) of hydroxylapatite (Biogel HTP, Bio-Rad Laboratories) equilibrated with the appropriate sonication buffer. The extract was washed on to the column with 20 to 30 ml of the same sonication buffer and proteins were then eluted with a linear phosphate gradient (10 to 400 mM, made up in 400 ml of the same sonication buffer).

Method 2: DEAE-cellulose chromatography. Crude cell-free extracts (20 to 25 ml) were applied directly to a column (5 × 4 cm) of DEAE-cellulose (Whatman DE 52) equilibrated with the appropriate sonication buffer. The extract was washed on to the column with 20 to 30 ml of the same sonication buffer and proteins were then eluted with a linear NaCl gradient (0 to 0.3 M, made up in 400 ml of the same sonication buffer).

Analytical methods and chemicals. Protein was estimated by the Lowry method using bovine serum albumin as standard. Biochemicals and enzymes were purchased from Boehringer, except transketolase, hydroxypyruvate, RiMP and XuMP which were obtained from Sigma. Formaldehyde was prepared from an aqueous suspension of paraformaldehyde by heating in a sealed ampoule at 110 °C for 16 to 24 h.

RESULTS

Assay of dihydroxyacetone synthase

O'Connor & Quayle (1980) described a continuous spectrophotometric assay for DHA synthase (reaction 1) which consisted of coupling the formation of DHA to the action of glycerol kinase (reaction 2) and glycerol-3-phosphate dehydrogenase (reaction 3):

Table 1. *Specific activities of DHA synthase and classical transketolase in crude extracts of C. boidinii grown on various substrates*

The assay method for DHA synthase was the discontinuous procedure (see Methods).

Growth substrate	Specific activity [$\mu\text{mol min}^{-1} (\text{mg protein})^{-1}$]	
	DHA synthase	Transketolase
Methanol	0.086	0.116
Glucose	0.007	0.098
Xylose	0.017	0.245
Ethanol	ND	0.060

ND, None detected.

Table 2. *Specific activities of DHA synthase and classical transketolase in crude extracts of glucose-grown C. boidinii after transfer to methanol growth medium*

Glucose-grown cells (4.5 g) of *C. boidinii* were harvested in mid-exponential phase, washed with sterile 20 mM-KH₂PO₄/NaOH buffer pH 7.1, and resuspended in 3 l methanol growth medium. Cultures were then incubated at 30 °C in shake flasks. The assay method for DHA synthase was the discontinuous procedure (see Methods).

Time after resuspension (h)	Specific activity [$\mu\text{mol min}^{-1} (\text{mg protein})^{-1}$]	
	DHA synthase	Transketolase
0	0.006	0.080
16	0.048	0.067
24	0.074	0.071

In order to correct for the presence of many interfering activities in crude extracts, each assay had to be performed twice with a different order of addition of reactants. The improved method described in the Methods section of the present paper obviates the need for duplicate assay with consequent saving of expensive reagents and time. Furthermore, by including triosephosphate isomerase in the assay mixture, the GAP produced in reaction (1) is isomerized to DHAP and is also detected by reaction (3), thus resulting in a twofold amplification of the rate of reaction (1) under conditions in which endogenous triosephosphate isomerase, originally present in the enzyme extract, may have been progressively removed during enzyme purification.

Synthesis of two transketolases during methylotrophic growth of C. boidinii

The specific activities of DHA synthase and classical transketolase were determined in crude extracts of *C. boidinii* grown on various substrates (Table 1). Appreciable DHA synthase activity was only detected in methanol-grown cells, whereas classical transketolase activity was found during growth on all substrates tested. Glucose-grown cells, containing an insignificant level of DHA synthase activity, when transferred to methanol growth medium showed increasing DHA synthase activity during the lag period of no growth, but classical transketolase activity remained relatively constant (Table 2). This indicated that the two activities were due to two different enzymes.

Fig. 1. Separation of two classical transketolase activities, one of which has DHA synthase activity, by chromatography of a crude extract of methanol-grown *C. boidinii* on hydroxylapatite. The extract (containing 100 mg protein) was passed through a column of hydroxylapatite using a linear phosphate gradient; details are given in Methods. Samples (50 μ l) of fractions (3.5 ml) were assayed for enzyme activity (results are expressed as μ mol min^{-1} in each fraction): ●, classical transketolase; ○, DHA synthase; —, phosphate gradient; ----, absorbance at 280 nm.

Fig. 2. Partial purification of classical transketolase from crude extracts of *C. boidinii* grown on various substrates. Extracts (containing 100 mg protein) were passed through separate columns of hydroxylapatite with identical linear phosphate gradients; details are given in Methods. Samples (20 to 50 μ l) of fractions (3.5 ml) were assayed for transketolase activity (results are expressed as μ mol min^{-1} in each fraction): ○, xylose-grown cells; ●, glucose-grown cells; □, ethanol-grown cells; —, phosphate gradient; ----, absorbance at 280 nm obtained with the extract from glucose-grown cells.

In previous studies (O'Connor & Quayle, 1980), DHA synthase activity was found to be very unstable, resulting in low yields during purification. In the present study, attempts to stabilize DHA synthase activity by the addition of compounds such as formaldehyde, EDTA, $(\text{NH}_4)_2\text{SO}_4$, methanol, ethanol, dithiothreitol, XuMP, and the protease inhibitor phenylmethylsulphonyl fluoride were unsuccessful. However, addition of 0.005% (w/v) TPP to all buffers used in the handling of the yeast extracts was found to have a slightly beneficial effect.

As large losses occurred at each stage of a purification, attempts were made to purify DHA synthase by direct chromatography of cell-free extracts on hydroxylapatite. Extracts of methanol-grown cells gave two peaks (A and B) of classical transketolase activity (Fig. 1), one of which (peak B) also contained DHA synthase activity. Extracts from cells grown on other substrates merely gave single peaks of classical transketolase activity, which were eluted from the columns at approximately the same position as the peak A from methanol-grown cells (Fig. 2). Thus, the peak containing both DHA synthase and classical transketolase

Fig. 3. Separation of two classical transketolase activities, one of which has DHA synthase activity, by chromatography of a crude extract of methanol-grown *C. boidinii* on DEAE-cellulose. The extract (containing 100 mg protein) was passed through a column of DEAE-cellulose (Cl⁻ form) using a linear NaCl gradient; details are given in Methods. Samples (50 μ l) of fractions (5 ml) were assayed for enzyme activity (results are expressed as μ mol min⁻¹ in each fraction): ●, classical transketolase; ○, DHA synthase; —, NaCl gradient; ----, absorbance at 280 nm.

Fig. 4. Thermal stability of the enzyme(s) contained in the combined fractions of peak B (see Fig. 1): ○, classical transketolase; ●, DHA synthase. Incubation temperature: a, 0 °C; b, 20 °C; c, 30 °C; d, 35 °C; e, 40 °C.

activities was only found in extracts of methanol-grown cells, whereas the peak containing classical transketolase activity alone was present in extracts of cells grown on all substrates tested, including methanol.

Attempts were made to separate the two activities of peak B by ion-exchange chromatography. Pooled fractions were dialysed and submitted to DEAE-cellulose chromatography. Major portions of both activities were lost during dialysis and neither activity was detected in eluate fractions from the ion-exchange column. A crude cell-free extract of methanol-grown cells was then applied directly to a DEAE-cellulose column and eluted with a linear NaCl gradient (Fig. 3). Two peaks of classical transketolase activity were detected; the first, and smaller, peak (C) also had DHA synthase activity in contrast to the larger peak (D) which did not. Extracts from glucose-grown cells produced only one peak of classical transketolase activity, which eluted in the same position as did peak D of extracts from methanol-grown cells.

In order to establish whether the two activities occurring together in peak B (Fig. 1) were due to the same enzyme, samples from the combined fractions of peak B were incubated at various temperatures (0 to 40 °C) and the two activities were monitored (Fig. 4). Classical transketolase and DHA synthase activities disappeared at the same rates at the five

Fig. 5. Thermal stability of classical transketolase (●), DHA synthase (○) and NADH oxidase (□) activities in crude extracts of methanol-grown *C. boidinii*, and classical transketolase (Δ) from glucose-grown *C. boidinii*, monitored during incubation at 35 °C (—) and 40 °C (----).

temperatures used. Activities were found to be relatively stable when maintained at 0 °C, but were totally lost within 10 or 30 min at 40 and 35 °C, respectively. Crude extracts of methanol-grown cells subjected to the same heat treatment lost approximately 50% of classical transketolase activity within 10 or 30 min at 40 and 35 °C, respectively, with accompanying total loss of DHA synthase activity (Fig. 5). The remaining classical transketolase activity was very stable, losing little further activity over the next hour. Over the whole period, NADH oxidase activity remained relatively constant, thus showing that non-specific protease activity was not responsible for the loss of enzyme activities.

In peaks B and C the ratios of DHA synthase activity to classical transketolase activity were approximately 1:2 and 1:1, respectively (see Figs 1 and 3). Classical transketolase activity was routinely assayed by addition of RiMP to an initial concentration of 5 mM in a system containing pentose phosphate-interconverting enzymes, resulting in an equilibrium mixture of RiMP:ribulose 5-phosphate:XuMP (75:25:75) (Tabachnick *et al.*, 1958) and thus an equilibrium concentration of approximately 2.1 mM for both RiMP and XuMP. The substrate concentrations of XuMP and formaldehyde used for the routine assay of DHA synthase were 2.5 mM and 1 mM, respectively. In order to compare the reactivities of the two alternative glycolaldehyde acceptors, namely RiMP and formaldehyde, DHA synthase and classical transketolase activities of the combined fractions of peak B were determined using assay concentrations of 2.5 mM for the glycolaldehyde donor (XuMP) and 1.0 mM for the acceptor (formaldehyde or RiMP). Under these conditions the ratio of activity with the two acceptors was approximately 1:1.

Hydroxypyruvate was also tested as an alternative glycolaldehyde donor, in place of XuMP, with formaldehyde as the acceptor. Rates with partially purified DHA synthase were approximately 35% of those obtained using equimolar XuMP. Tani *et al.* (1980) have reported similar results with the partially purified DHA synthase from *Kloeckera* sp. 2201.

The classical transketolase peaks (A and D) observed in extracts from methanol-grown cells (Figs 1 and 3) and the single peaks found during growth on other substrates (Fig. 2) did not exhibit appreciable DHA synthase activity, but low rates might be expected since Dickens & Williamson (1958) showed that in the presence of purified classical transketolase from baker's yeast, formaldehyde slowly accepts a glycolaldehyde fragment donated by hydroxypyruvate. In these experiments 500 µg transketolase in 2.5 ml reaction mixture catalysed a rate of reaction between these substrates of 15 to 18 µmol over 2 h incubation at 37 °C. Without a progress curve for the reaction it is not possible to establish the specific activity of the enzyme from these data. We therefore tested the highly purified classical transketolase from baker's yeast (Sigma) for DHA synthase activity using our own

continuous spectrophotometric assay with 2.5 mM-XuMP and 1 mM-formaldehyde as substrates. A rate of $0.245 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$ was observed which was 1% of the rate found when RiMP replaced formaldehyde as acceptor. If the classical transketolase from *C. boidinii* behaves similarly to that from baker's yeast, and there is no reason to suppose otherwise, it can be calculated that the activity of the former enzyme with formaldehyde as acceptor could not have been detected by the continuous spectrophotometric assay in samples of the appropriate peaks taken from the columns described in Figs 1, 2 and 3 without considerable further purification and concentration of the enzyme contained therein.

DISCUSSION

These results show that *C. boidinii* can synthesize two transketolases: (a) an unstable enzyme exhibiting both DHA synthase and classical transketolase activities; (b) a relatively stable enzyme exhibiting only classical transketolase activity. The former enzyme is only synthesized during growth on methanol, whereas the latter enzyme is synthesized during growth on all substrates tested, including methanol. It is thus clear that measurements of classical transketolase activity (using XuMP as donor and RiMP as acceptor) in crude extracts of a methylotrophic yeast can give a composite figure for two enzymes depending on the growth conditions. A convenient method for separating this composite figure into its two components (in *C. boidinii* at least) would be first to measure the DHA synthase activity with XuMP and formaldehyde, and then to heat the extracts for 10 min at 40 °C or 30 min at 35 °C before measuring the classical transketolase activity with XuMP and RiMP.

It is not clear at this stage why in the previous study of O'Connor & Quayle (1980) classical transketolase activity was not detected in the peak of DHA synthase activity obtained by DEAE-cellulose chromatography. Due to heavy losses of enzyme activity during the desalting on Sephadex G-25 and chromatography on DEAE-cellulose, the levels of DHA synthase that were finally assayed by these authors were very low and hence assays were not as accurate as in the one-step separation procedure developed in the present study. The greater accuracy of assay that is attainable with greater enzyme activities may indeed be one factor involved; however, this may not be the primary factor. During the present study it was observed that in peaks exhibiting dual activity of DHA synthase and classical transketolase, the ratio of the former activity to the latter varied from approximately 1:2 to 1:1 depending upon the method of purification (compare peak B in Fig. 1 with peak C in Fig. 3). This suggests that certain treatments of the enzyme, e.g. ion-exchange chromatography, can alter its relative reactivity towards formaldehyde and RiMP as glycolaldehyde acceptors. It should also be noted that passage of a crude extract through a DEAE-cellulose column caused much greater losses of DHA synthase activity than did a similar passage through a hydroxylapatite column. Further work is needed to explore this.

It has long been known that the classical transketolase of baker's yeast can slowly utilize hydroxypyruvate as glycolaldehyde donor and formaldehyde as acceptor (Dickens & Williamson, 1958). We have further shown that, as might be expected, this enzyme also exhibits a low DHA synthase activity of approximately 1% of its classical transketolase activity. Similarly, it has been found that hydroxypyruvate can replace XuMP as a glycolaldehyde donor for the DHA synthase found in methanol-grown *C. boidinii*.

An interesting problem is raised by the high classical transketolase activity displayed by DHA synthase. Does the former activity play any physiological role during growth on methanol? Classical transketolase is expected to play a key role in the XuMP cycle of formaldehyde fixation during the regeneration of pentose phosphate from fructose phosphate and triose phosphate (van Dijken *et al.*, 1978). Can DHA synthase assume this role as well as that of formaldehyde fixation, or is it left to the second classical transketolase which is present under all growth conditions? Mutant studies may help to answer this question.

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