Inhibition of transketolase by p-hydroxyphenylpyruvate

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Abstract The effect of *p*-hydroxyphenylpyruvate, a natural analogue of transketolase substrate, on the catalytic activity of the enzyme was investigated. *p*-Hydroxyphenylpyruvate proved to be a reversible and competitive inhibitor of transketolase with respect to substrate; it was also able to displace thiamine diphosphate from holotransketolase. The data suggest that *p*-hydroxyphenylpyruvate participates in the regulation of tyrosine biosynthesis by influencing the catalytic activity of transketolase.
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Key words: Hydroxyphenylpyruvate; Regulation; Thiamine diphosphate; Transketolase

1. Introduction

Transketolase (TK; D-sedoheptulose 7-phosphate: D-glyceraldehyde-3-phosphate glycolaldehydetransferase, EC 2.2.1.1) is the key enzyme of the non-oxidative part of the pentose-phosphate pathway [1]. Together with transaldolase, it provides a link between the pentose-phosphate pathway and the glycolytic one, enabling the recycling of pentose sugars under conditions where NADPH production is required for reductive biosynthesis. TK is indispensable for generation of erythrose 4-phosphate which is needed for the biosynthesis of aromatic amino acids; in addition, the enzyme is essential for generation of ribose 5-phosphate which is utilized in the biosynthesis of nucleic acids and some coenzymes [2,3]. TK catalyzes cleavage of a C–C bond in ketose (donor substrate) and subsequent transfer of the two-carbon fragment to aldose (acceptor substrate). The TK reaction is reversible except when hydroxypyruvate (HP) is used as a donor substrate. Thiamine diphosphate (ThDP) and bivalent cations, such as Ca^{2+}, Mg^{2+}, are TK cofactors [4].

The molecular properties of the enzyme from baker's yeast have been studied in sufficient detail [5–8]. Its amino acid sequence, some functional groups of the apoprotein and the coenzyme, as well as the enzyme's crystal structure and organization of its active site, are already known. Some progress has been made towards the understanding of the mechanism of TK action. However the mechanism of regulation of the enzyme's catalytic activity remains unclear.

The present experiments were undertaken to investigate the influence of *p*-hydroxyphenylpyruvate (HPP) on the activity of TK from baker's yeast, since HPP is known to be both a natural cellular metabolite and the analogue of the donor substrate of TK. We found that HPP acts as a reversible and competitive inhibitor with respect to donor substrate of TK and exerts a significant influence on the dissociation of holoTK into the apoenzyme and coenzyme. It was suggested that HPP is a regulator of the enzyme activity in vivo.

2. Materials and methods

2.1. Materials

ThDP and dithiothreitol were purchased from Merck, NAD^+ from Reanal, Sephadex G-50 from Pharmacia, HP, HPP, fructose 6-phosphate (F6P) and ribose 5-phosphate from Sigma, glycolaldehyde from Serva. A mixture of phosphopentoses, used as a substrate for the transketolase activity measurements, was obtained from ribose 5-phosphate [9].

The solution of the ketonic form of HPP was prepared using the method described in [10] with slight modifications: the HPP concentration was 200 mM instead of 0.56 mM and the incubation time at pH 9.0 was 10 min instead of 20 min. The production of the ketonic from the enolic form of HPP was controlled spectrophotometrically [11]. The resulting solution of the ketonic form of HPP was stored at pH 7.4.

2.2. TK purification

Transketolase was isolated from baker's yeast according to the method described earlier [12]. The crystalline enzyme was stored at 4°C in $(NH_4)_2SO_4$ solution of 0.5 saturation, pH 7.6. TK was homogeneous, according to the data of SDS-Na-electrophoresis; its specific activity was 13 U/mg. The TK concentration was determined using the absorbance coefficient $A_{280}^{1\%} = 14.5$ [13]. The apoenzyme was obtained by keeping 2 mg/ml of TK in 1.6 M ammonium sulfate, pH 7.6, for 48 h. Prior to use, the TK solution was passed through a Sephadex G-50 column to remove ammonium sulfate.

2.3. TK activity

The catalytic activity of TK was measured spectrophotometrically at 25°C (Cary 219 spectrophotometer) by the rate of NAD^+ reduction in a coupled system with glyceraldehydephosphate dehydrogenase [4]. The reaction mixture (final volume 0.6 ml) contained: 50 mM glycylglycine, 5 mM sodium arsenate, 0.25 mM NAD^+, 3 U glyceraldehydephosphate dehydrogenase, 3 mM dithiothreitol, 2.5 mM $CaCl_2$, 0.1 mM ThDP, 7 mM phosphopentoses (xylulose 5-phosphate+ribose 5-phosphate); pH 7.6. The reaction was initiated by TK addition.

2.4. Determination of K_m for the donor substrates of TK

To determine the values of K_m for HP (A) and F6P (B) in the absence and in the presence of HPP (Fig. 1), the TK activity was measured by the oxidation rate of the α-carbanion intermediate in the presence of ferricyanide [14] (A) and by the amount of sedoheptulose 7-phosphate formed [4] (B). The reaction mixture contained in a final volume of 0.6 ml: (A) 50 mM glycylglycine, 2.5 mM $MgCl_2$, 0.1 mM ThDP, 4 mM $K_3[Fe(CN)_6]$, 3.3 mM (2) or 5.2 mM (3) HPP, 0.31 µM TK and varying HP; pH 7.6; (B) 50 mM glycylglycine, 2.5 mM $MgCl_2$, 0.04 mM ThDP, 5 mM ribose 5-phosphate, 3.0 mM (2) or 5.0 mM (3) HPP; 0.18 µM TK and varying F6P; pH 7.6.

The K_i for HPP relative to the substrates was calculated by the equation:

$$K_i = [i]/\{(K_m^i/K_m)-1\}$$

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where K_m and K_m^i are Michaelis constants in the absence and presence of the inhibitor, respectively.

2.5. HPP influence on the reconstitution of holoTK

For estimating the influence of HPP on the reconstruction rate and on the level of holoTK formation (Fig. 2), apoTK was preincubated with ThDP at 25°C in 50 mM glycylglycine buffer, pH 7.6, in the absence or in the presence of HPP (HPP was added 5 min before ThDP). At intervals, aliquots of 5–10 μl were added to the reaction mixture (free from ThDP) and the enzyme activity was measured. The measured activity corresponds to the amount of holoTK formed during reconstruction.

2.6. Influence of HPP on holoTK activity

To study the influence of preincubation of holoTK plus HPP on the enzyme activity (Fig. 3), apoTK (0.62 μM) was preincubated for 30 min in 50 mM glycylglycine buffer, pH 7.6, containing 2.5 mM CaCl₂ and 5.5 μM ThDP. The coenzyme concentration of 5.5 μM was the minimal one at which apo- to holoenzyme conversion could be attained. After holoenzyme formation, the preincubation (with and without HPP) was continued. At intervals, aliquots of 5–10 μl were taken to measure the TK activity without addition of ThDP.

2.7. Influence of HPP on the coenzyme concentration dependence of TK activity

ApoTK was preincubated at 25°C in 50 mM glycylglycine buffer, pH 7.6, with 2.5 mM CaCl₂ at varying ThDP concentrations, in the absence or in the presence of HPP. Thirty minutes later (the time necessary to reach the equilibrium of apoTK+ThDP ⇌ holoTK with the minimal coenzyme concentration used in our experiments), aliquots of 5–10 μl were added to the reaction mixture (free from ThDP) to measure the enzyme activity.

3. Results and discussion

3.1. Inhibition of TK by HPP

HPP inhibited TK activity in a concentration-dependent

Fig. 2. The influence of HPP on the reconstitution of holoTK from apoTK and coenzyme. The preincubation medium: 50 mM glycylglycine, 0.62 μM apoTK, 2.5 mM CaCl₂, 5.5 μM ThDP, 0.0 (1), 10 (2), 20 (3) and 35 mM (4) HPP; pH 7.6. Aliquots from the preincubating samples were taken at certain periods of time for measuring the enzymatic activity as described in Section 2 but without ThDP.

manner. The inhibition was reversible, and HPP removal led to complete recovery of TK activity. Fig. 1 shows that HPP is a competitive inhibitor with respect to donor substrate, HP or F6P. K_i values obtained with the two substrates were similar: 2.5 and 3.0 mM for HP and F6P, respectively. As will be shown below, HPP is able to displace ThDP from the holoTK molecule. It is noteworthy that in experiments displayed in Fig. 1, TK was always present in the form of holoenzyme (data not shown). Therefore the curves show only the competitive effect, with respect to substrate, of the inhibitor. Special experiments revealed that HPP is not a TK substrate (data not shown).

3.2. Influence of HPP on the dissociation of holoTK into the apo- and coenzymes

In this set of experiments, holoTK was determined by the level of its catalytic activity. The HPP concentration in the reaction mixture used for measuring the enzyme activity was so low that HPP could not influence the TK activity, while competing with substrate.
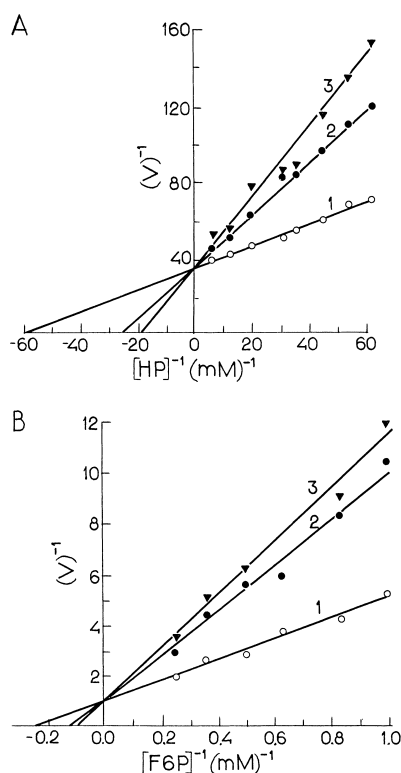
Fig. 1. Determination of K_m for HP (A) and F6P (B) in the absence (1) and in the presence (2, 3) of HPP. For conditions of the experiments see Section 2.
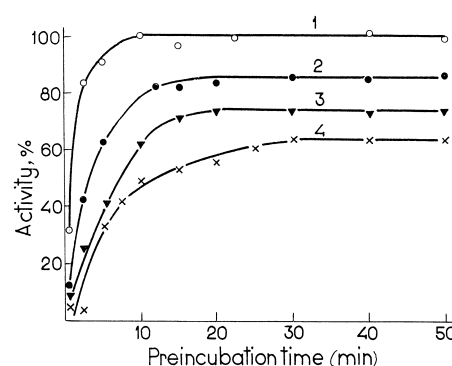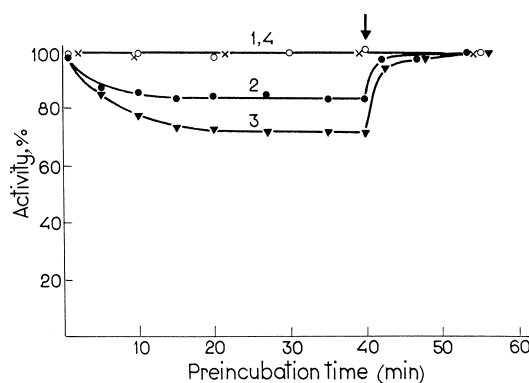
Fig. 3. The influence of preincubation of holoTK with HPP on the activity of holoTK. The preincubation medium: 50 mM glycylglycine, 0.62 μM apoTK, 2.5 mM CaCl₂, 5.5 μM ThDP; 1, without HPP; 10 mM (2) or 20 mM (3) HPP; 35 mM HPP+12 mM HP (4); pH 7.6. The arrow indicates the time when 0.5 mM ThDP was added to all preincubating samples. The activity in the aliquots taken from the preincubating samples was measured as described in Section 2 but without ThDP. The details of the experiment are shown in Section 2.
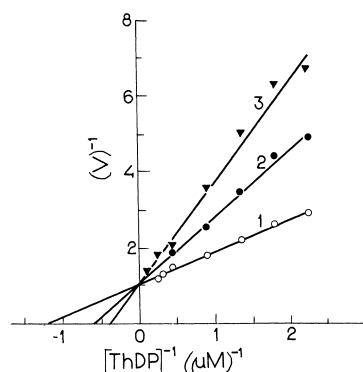
Fig. 4. Influence of HPP on the ThDP concentration dependence of TK activity. For conditions of the experiments see Section 2.

It is known that the rate of holoTK reconstitution from apoTK and coenzyme depends on the coenzyme concentration [15,16]. The reconstitution of holoTK was followed by measuring its activity (Fig. 2, curve 1). The coenzyme concentration used allowed us to monitor the reconstitution process (with excess ThDP, holoenzyme formation occurs so quickly that it cannot be registered experimentally [15,16]). The concentration was sufficient to obtain the holoenzyme in 100% yield. In the presence of HPP, both the reconstitution rate and the amount of holoTK formed decreased in a concentration-dependent manner (curves 2–4). The data show that ThDP and HPP compete between themselves for the binding site on the enzyme surface.

Preincubation of holoTK with HPP led to a gradual decrease in the enzyme's catalytic activity until it reached a constant level. The degree of inhibition depended on HPP concentration (Fig. 3, curves 1–3). Subsequent addition of ThDP to the preincubation mixture (indicated by the arrow) fully restored the TK activity. Therefore HPP is able to displace ThDP from the TK molecule and vice versa.

The fact that preincubation of holoTK with HPP in the presence of HP (donor substrate of TK) does not decrease the enzyme activity (Fig. 3, curve 4) seems to support the view that HPP is able to displace ThDP from holoTK. It is known that the action of TK causes irreversible decarboxylation of HP; as a result, the apoprotein-bound dihydroxyethylThDP is formed, whose affinity to the enzyme is about 100-fold higher than that of ThDP [17]. In light of these data it becomes clear why HPP displaces ThDP from holoTK (Fig. 3, curves 2 and 3), competing with the coenzyme for the apoprotein, but is unable to displace dihydroxyethylThDP from its complex with the protein (Fig. 3, curve 4).

Further evidence for the competition between HPP and the coenzyme was obtained in experiments the results of which are presented as double reciprocal plots of holoTK formation (as measured by the TK activity level) vs. ThDP concentration

(Fig. 4). One can see that all the straight lines obtained both in the absence and in the presence of HPP intersect with the ordinate at the same point, attesting to the competition between ThDP and HPP for apoTK.

4. Conclusions

The experimental data presented herein indicate that HPP is a reversible and competitive (with respect to donor substrate) inhibitor of TK. It is known that HPP is a natural cellular metabolite, a precursor of tyrosine. It converts to tyrosine during the transamination reaction. The initial stage of tyrosine biosynthesis involves condensation of phosphoenolpyruvate with erythrose 4-phosphate, formed from F6P (donor substrate) under the action of transketolase. The affinities of HPP and F6P to TK, as determined in our experiments, are nearly the same: the K_i for HPP is 3.0 mM and the K_m for F6P is 4.0 mM (Fig. 1B). There is reason to believe that the inhibition of TK by HPP is one of the possible mechanisms for the tyrosine biosynthesis regulation.

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