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Halogenated Pyruvate Derivatives as Substrates of Transketolase from *Saccharomyces cerevisiae*

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Abstract—Pyruvate derivatives halogenated at C3 were shown to be donor substrates in the transketolase reaction. No drastic differences between the derivatives were observed in the value of the catalytic constant, whereas the Michaelis constant increased in the following order: Br-pyruvate < Cl-pyruvate < Cl_2-pyruvate < F-pyruvate < Br_2-pyruvate. The presence of the halogenated pyruvate derivatives increased the affinity of apotransketolase for the coenzyme; of note, the extent of this effect was equal with both of the active centers of the enzyme. In contrast, the presence of any other substrate known to date, including hydroxypyruvate (i.e. pyruvate hydroxylated at C3), induced nonequivalence of the active centers in that they differed in the extent to which the affinity for the coenzyme increased. Consequently, the β-hydroxyl of dihydroxyethylthiamine diphosphate (an intermediate of the transketolase reaction) played an important role in the phenomenon of nonequivalence of the active centers associated with the coenzyme binding. The fundamental possibility was demonstrated of using halogenated pyruvate derivatives as donors of the halogen-hydroxyethyl group in organic synthesis of halogenated carbohydrates involving transketolase.

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Transketolase (TK, EC 2.2.1.1), an enzyme of the pentose-phosphate pathway of carbohydrate transformation, catalyzes the cleavage of ketoses (donor substrates) and the transfer of the resulting two-carbon fragment (glycolaldehyde residue) to aldose (acceptor substrate) [1, 2]. TK, a homodimer with molecular weight 148.4 kDa, has two active centers [3-6]. Thiamine diphosphate (ThDP) serves as a coenzyme. For the enzyme to be active, bivalent metal ions are also required (Ca^{2+}, Mg^{2+}, Mn^{2+}, etc.) [7]. Only Ca^{2+} was found in the native enzyme (2 g-atoms per mol protein, i.e. one atom in each active center) [8]. This cation is involved in the formation of the active center, bridging the pyrophosphate group of the coenzyme and the apoprotein. Substituting other bivalent cations for Ca^{2+} does not affect the catalytic activity substantially, even though it changes both TK conformation and its kinetic characteristics [9, 10].

The interaction of ThDP with apoTK occurs in two stages:

$$TK + ThDP \leftrightarrow TK\cdots ThDP \leftrightarrow TK^*{-}ThDP.$$

The first stage is rapid and readily reversible. It results in the formation of the catalytically inactive complex TK···ThDP, which then undergoes conformational changes. This second stage, slow and quasi-irreversible, produces the catalytically active holoenzyme, TK*–ThDP [11, 12].

In the presence of Ca^{2+}, the active centers of TK exhibit negative cooperativity with respect to ThDP (ThDP binding to one of the two active centers causes the other active center to decrease its affinity for the coenzyme by approximately an order of magnitude) [10, 12,

Abbreviations: HP, hydroxypyruvate; ThDP, thiamine diphosphate; TK, transketolase.

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13]. Substituting Mg^{2+} for Ca^{2+} either eliminates this nonequivalence [14, 15] or attenuates it [10].

Donor substrates increase the affinity of apoTK for ThDP, but the extent to which this effect is pronounced varies between the two active centers. For this reason, marked nonequivalence of the active centers induced by coenzyme binding is also observed in the presence of Mg^{2+} [16]. The effect of donor substrates is due to their cleavage and the resulting formation of dihydroxyethyl-ThDP (the main intermediate of the transketolase reaction), which interacts with TK with a higher affinity than ThDP.

Donor substrates of TK are characterized by the presence of (a) an oxo group adjacent to the C–C bond undergoing cleavage, (b) a hydroxyl at C1, and (c) *trans*-configuration of hydroxyls at the asymmetric C3 and C4 [2]. Dihydroxyacetone and hydroxypyruvate (HP), which have no asymmetric carbon atoms, constitute an exception. Requirements to be met by acceptor substrates (aldehydes) are by no means that strict: a considerable number of diverse aldehydes (including aromatic and heterocyclic) are susceptible TK [17, 18]. TK exhibits a higher affinity for phosphorylated than non-phosphorylated substrates, but this difference does not extend to catalytic activities observed in either case [19].

The transketolase reaction is reversible except when HP serves as the donor substrate. The irreversibility of the transketolase reaction with HP as a substrate and the formation of CO_2 as a reaction product in this case account for the feasibility of using TK in organic synthesis (of carbohydrates in particular) [17, 18, 20, 21].

Phosphorylated sugars are typical substrates of TK; examples include xylulose 5-phosphate, fructose 6-phosphate, and sedoheptulose 7-phosphate (donor substrates), and ribose 5-phosphate and glyceraldehyde-3-phosphate (acceptor substrates). Hydroxypyruvate is regarded as an atypical substrate of TK, hence its use as a tool in the studies of the enzyme. Pyruvate cannot function as a substrate in the transketolase reaction. The only difference of HP from pyruvate (which also accounts for the ability to serve as a donor substrate) is the presence of a hydroxyl, rather than hydrogen, at C3.

This goal of this work was to study halogenated pyruvate derivatives as potential donor substrates of TK.

MATERIALS AND METHODS

Chemicals used in this work included ThDP, Mops, and glycylglycine from Serva (Germany), and NAD^+, rabbit muscle glyceraldehyde-3-phosphate dehydrogenase, $CaCl_2$, β-hydroxypyruvate (Li salt), and $MgCl_2$ from Sigma Aldrich Chemie GmbH (Germany). Other reagents, from domestic manufacturers, were special purity or reagent grade. Halogenated pyruvate derivatives were the courtesy of Dr. R. Golbik (Martin-Luther-University Halle-Wittenberg, Institute of Biochemistry/Biotechnology, Halle/Saale, Germany).

Preparation of transketolase. Recombinant TK (as an apoenzyme) was obtained from the yeast *Saccharomyces cerevisiae* as described previously [22]. The enzyme was homogeneous by SDS-PAGE. The concentration of TK was determined spectrophotometrically ($A^{1\%}_{1\,cm}$ = 14.5 at 280 nm) [23]. The specific activity of the preparation was 22 U/mg.

Measurement of transketolase activity. TK activity was measured spectrophotometrically by the rate of NAD^+ reduction using glyceraldehyde-3-phosphate dehydrogenase as an auxiliary enzyme [1]. In determining the values of K_m for the substrates, the activity was measured in a system containing ferricyanide, an artificial electron acceptor [24]. Ferricyanide oxidizes the carbanion intermediate of the transketolase reaction at the intermediate stage of donor substrate conversion. The decrease in absorption caused by ferricyanide reduction was recorded at 420 nm. All measurements were performed using an Aminco DW 2000 (USA) spectrophotometer (double-beam mode). The reaction mixture contained (final volume, 2 ml): 50 mM Mops, pH 7.6, 2.5 mM $MgCl_2$, 0.1 mM ThDP, 1.25 mM $K_3[Fe(CN)_6]$, and an appropriate concentration of HP or a halogenated pyruvate derivative. The reaction was initiated by adding 50 to 60 μg TK. No TK was added into the reference cuvette.

Measurement of ThDP concentration. The concentration of ThDP was determined spectrophotometrically using the molar extinction coefficient $\varepsilon = 7500\ M^{-1}\cdot cm^{-1}$ at $\lambda = 273.5$ nm [25].

Measurement of K_d for ThDP. ThDP binding to apoTK and the formation of the catalytically active holoenzyme is accompanied by the appearance in the spectrum of a new band with maximum at 320 nm, the intensity of which correlated with the amount of the coenzyme bound to the active centers of TK [5, 6]. The apoenzyme (2.1 mg apoTK in 50 mM glycylglycine buffer, pH 7.6, supplemented with 2.5 mM $MgCl_2$) was introduced into a spectrophotometric cell (total volume, 3 ml). The original absorption at 320 nm was recorded, followed by addition of 10 μl ThDP (2.3 μM). Subsequent additions were made on completion of absorption measurements. The lack of absorption changes after addition of the last portion of the ThDP solution indicated that complete binding of the coenzyme occurred at the two active centers. In determining the effects of the substrates on holoTK reconstruction, the substrates were introduced into the reaction mixture prior to ThDP addition. Changes in the absorption were recorded on the Aminco DW 2000 spectrophotometer using the dual-wavelength mode (λ_1 = 320 nm, λ_2 = 400 nm).

The values of the dissociation constant for each of the two active centers were calculated based on the data of spectrophotometric titration. Maximum change in the

absorption at the saturating concentration of ThDP corresponded to maximum (100%) formation of holoTK. The value of K_d for ThDP was calculated using SCIENTIST software and the equation describing the interaction of the coenzyme with the two active centers:

$$[\text{holoTK}] = \frac{0.5\,[\text{TK}]\,[\text{ThDP}_f]}{[\text{ThDP}_f] + K_d^1} + \frac{0.5[\text{TK}]\,[\text{ThDP}_f]}{[\text{ThDP}_f] + K_d^2}.$$

The concentration of the free coenzyme ($ThDP_f$) was determined using the following equation:

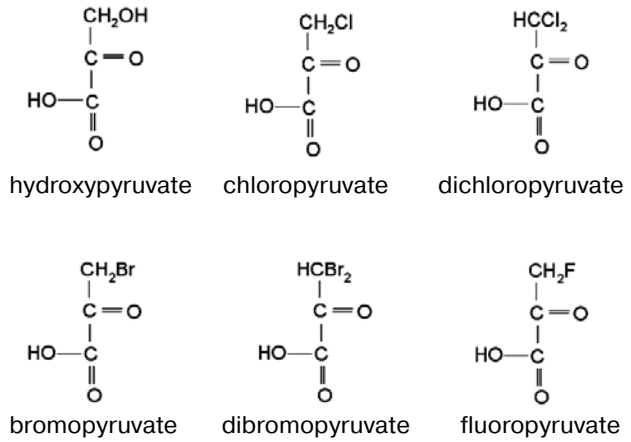
$$[\text{ThDP}_f] = [\text{ThDP}_{\text{total}}] - [\text{ThDP}_b],$$

where $[ThDP_b]$ is the concentration of active centers that bound ThDP.

Circular dichroism spectra were recorded at 260-400 nm and 20°C using a PC-operated Jobin Yvon (France) dichrograph (Jasco J810 or Mark V) equipped with a 1-cm cell. The reaction mixture contained 25 mM glycylglycine buffer, pH 7.6, 0.79 mg/ml TK, 30 μM ThDP, 2.5 mM $CaCl_2$, 2 mM HP (or an appropriate concentration of a halogenated pyruvate derivative), and 20 mM glycolaldehyde.

RESULTS AND DISCUSSION

Hydroxypyruvate (see Scheme), a hydroxylated derivative of pyruvate, is a donor substrate of TK. Halogenated pyruvate derivatives (see Scheme) can be viewed as analogs of hydroxypyruvate in which the hydroxyl group is replaced by a halogen. For this reason, we always run parallel experiments with HP to be able to compare the data.



Structure of donor substrates of transketolase (HP and halogenated pyruvate derivatives)

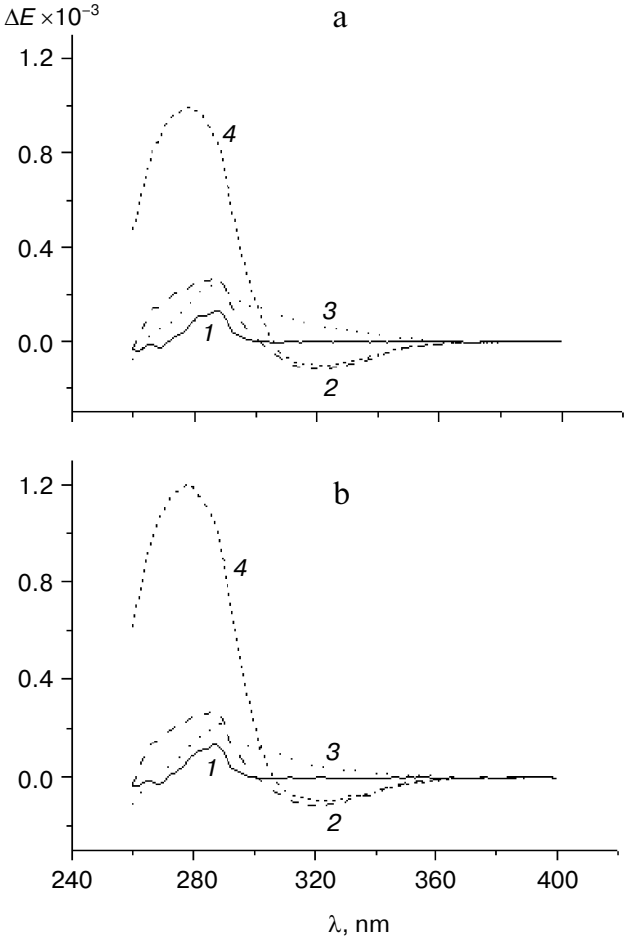


Fig. 1. CD spectra of transketolase taken in the presence of HP (a) or Br-pyruvate (b) as donor substrates. a) 25 mM glycylglycine buffer, pH 7.6, 0.79 mg/ml TK, 30 μM ThDP, 2.5 mM $CaCl_2$, 20 mM glycolaldehyde, 2 mM HP. b) 25 mM glycylglycine buffer, pH 7.6, 0.79 mg/ml TK, 30 μM ThDP, 2.5 mM $CaCl_2$, 20 mM glycolaldehyde, 4 mM Br-pyruvate.

Catalytic conversions of halogenated pyruvate derivatives by transketolase. Binding of ThDP to apoTK induces the appearance in its CD spectrum of an absorption band with a maximum at 320 nm that was lacking in the initial components (spectrum *2* in Fig. 1a). This band reflects the formation of the active center and the assembly of the catalytically active holoenzyme [26]. A clear correlation has been observed between the intensity of the induced absorption band and the amount of holoTK formed [5, 6]. The band disappears on addition of donor substrate. If the donor substrate is cleaved irreversibly, such as HP, an inversion of this band occurs as exemplified by spectrum *3* in Fig. 1a. The reason for this change in the spectrum of holoTK is the cleavage of the keto substrate and the formation of dihydroxyethyl-ThDP (the main intermediate of the transketolase reac-

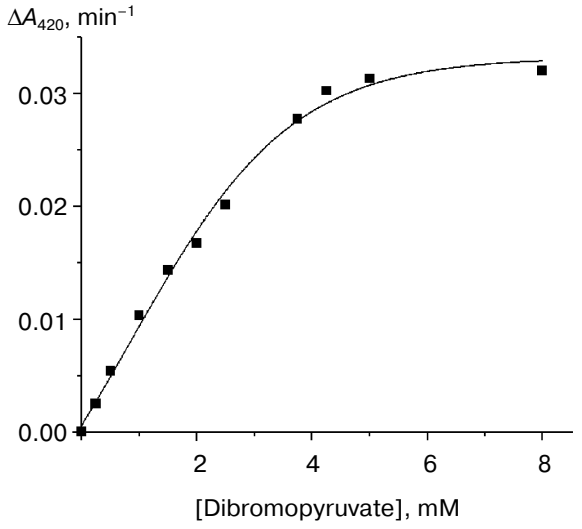


Fig. 2. Dependence of holoTK activity on the concentration of dibromopyruvate (50 mM Mops, pH 7.6, 2.5 mM $CaCl_2$, 0.15 mM ThDP, 1.25 mM $K_3[Fe(CN)_6]$, 50 μg/ml TK).

tion) during the first stage of the catalytic process. In the second stage of the transketolase reaction, the dihydroxyethyl residue is transferred to the acceptor substrate, which is accompanied by recovery of the original appearance of the spectrum of holoTK (spectrum *4* in Fig. 1a).

The use of Br-pyruvate as a donor substrate is associated with the same pattern of changes in the spectrum of holoTK as that observed with HP (compare Figs. 1a and 1b). In this case, the transketolase reaction also occurred in two stages. The spectral changes of holoTK induced by other halogenated pyruvate derivatives (Cl-pyruvate, Cl_2-pyruvate, F-pyruvate, Br_2-pyruvate) exhibited no significant differences from those depicted in Fig. 1b (data not presented).

Thus, all halogenated pyruvate derivatives studied in this work are capable of functioning as donor substrates in the transketolase reaction.

Determination of K_m values for halogenated pyruvate derivatives. Figure 2 presents data on the effect of dibromopyruvate concentration on the catalytic activity of TK. In this case, the activity was determined by the oxidation rate of dihydroxyethyl-ThDP (the main intermediate of the transketolase reaction) measured in the presence of the artificial electron acceptor ferricyanide. The hyperbolic dependence obtained reflects equivalent affinities of the two active centers of TK for the substrate. Similar concentration dependences were obtained for the activity of the enzyme with other halogenated pyruvate derivatives and HP (data not presented). The table lists the kinetic characteristics derived from experimental data on the effects of donor substrates on the catalytic activity of TK.

Bromopyruvate was the most active among monosubstituted halogenated substrates, even exceeding the activity of HP. With chloropyruvate the activity was somewhat lower. Fluoropyruvate was the least active (the activity of TK was three times lower with this substrate than HP). Rank order of efficiency of monosubstituted halogenated pyruvate derivatives as TK substrates had the following appearance: Br-pyruvate > OH-pyruvate > Cl-pyruvate > F-pyruvate. In general, however, all values obtained were comparable. With dihalogenated pyruvate derivatives, TK exhibited the same activity as that measured in the presence of HP.

Out of all substrates used in this work, HP was characterized by the lowest K_m value. Substituting bromine for hydroxyl caused a slight increase in K_m; with chlorine and fluorine, the respective values increased 10 and 50 times (table). When arranged by K_m values, the studied substrates formed the following rank order: OH-pyruvate < Br-pyruvate < Cl-pyruvate < Cl_2-pyruvate < Br_2-pyruvate < F-pyruvate.

Effects of HP and halogenated pyruvate derivatives on ThDP binding to apoTK. Figure 3 shows the results of experiments in which the effect of HP on the interaction of ThDP with apoTK was assessed by spectrophotometric titration. In the absence of the substrate, the affinities of the active centers for the coenzyme were equal: K_d^1 =

Kinetic characteristics of HP and halogenated pyruvate derivatives as donor substrates

Substrate	k_{cat}, %	K_m ($S_{0.5}$), mM	K_d^1 (ThDP), μM	K_d^2 (ThDP), μM
Without substrate	–	–	5.2	5.2
Hydroxypyruvate	100	0.05	< 0.01	1.6
Bromopyruvate	130	0.13	0.6	0.6
Dibromopyruvate	90	2.0	–	–
Chloropyruvate	80	0.4	1.5	1.5
Dichloropyruvate	100	1.1	4.4	4.4
Fluoropyruvate	30	2.5	–	–

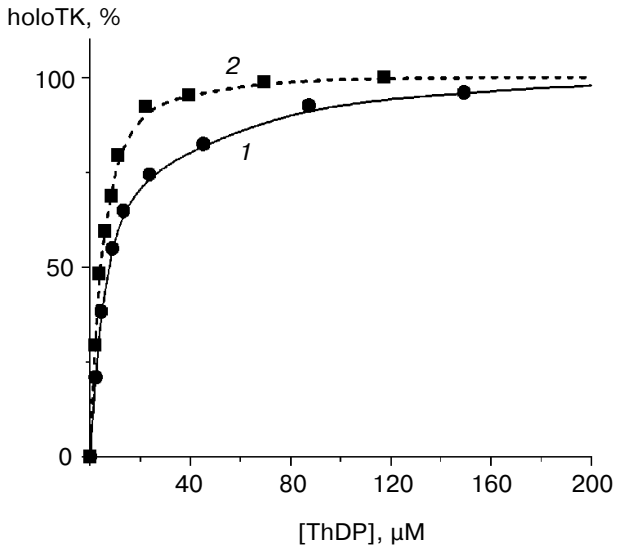


Fig. 3. Effect of ThDP concentration on the amount of holoTK formed in the absence (*1*) and in the presence (*2*) of 2.5 mM HP (50 mM glycylglycine buffer, pH 7.6, 2.5 mM $MgCl_2$, 3.4 μM TK). Dots represent experimental data, the curves are plotted using the parameters in the table.

K_d^2 = 5.2 μM (table). In the presence of HP, the affinity for ThDP increased for both active centers; most importantly, the two centers differed in the extent of this increase. For one center, K_d^2 decreased to 1.6 μM; for the other, the increase in affinity was so pronounced that it could not be assessed by the method used (all coenzyme added was stoichiometrically bound to the first active center). Thus, in the presence of HP the affinity of both centers for ThDP increased and their nonequivalence was induced (negative cooperativity of coenzyme binding).

Similar experiments run with halogenated pyruvate derivatives demonstrated that, in the presence of dibromopyruvate, the affinity of apoTK for ThDP undergoes insignificant changes, if any. With chloropyruvate and, particularly, bromopyruvate, a marked decrease in the values of the dissociation constant was observed. It is important that the increase in the affinity for these substrates did not vary between the active centers: the values of the dissociation constant obtained for the first and second active center were equal (table). Thus, halogenated substrates increase the affinity of the active centers of TK for ThDP without inducing their nonequivalence, in contrast to HP. It would follow that the β-hydroxyl group of dihydroxyethyl-ThDP, the main intermediate of the transketolase reaction, plays an important role in the phenomenon of active center nonequivalence with respect to coenzyme binding.

In conclusion, halogenated pyruvate derivatives can function as donor substrates of TK and be used for organic synthesis of halogenated carbohydrate derivatives.

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