ISSN 0006-2979, Biochemistry (Moscow), 2009, Vol. 74, No. 7, pp. 789-792. © Pleiades Publishing, Ltd., 2009.
Original Russian Text © I. A. Sevostyanova, V. A. Selivanov, V. A. Yurshev, O. N. Solovjeva, S. V. Zabrodskaya, G. A. Kochetov, 2009, published in Biokhimiya, 2009, Vol. 74, No. 7, pp. 972-976.

Cooperative Binding of Substrates to Transketolase from *Saccharomyces cerevisiae*

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Received July 17, 2008
Revision received October 6, 2008

Abstract—Catalytic activity of two active sites of transketolase and their affinity towards the substrates (xylulose-5-phosphate and ribose-5-phosphate) has been studied in the presence of Ca^{2+} and Mg^{2+}. In the presence of Ca^{2+}, the active sites exhibit negative cooperativity in binding both xylulose-5-phosphate (donor substrate) and ribose-5-phosphate (acceptor substrate) and positive cooperativity in the catalytic transformation of the substrates. In the presence of Mg^{2+}, nonequivalence of the active sites is not observed.

DOI: 10.1134/S0006297909070128

Key words: transketolase, nonequivalence of active sites, bivalent cations, xylulose-5-phosphate, ribose-5-phosphate

Transketolase (TK, EC 2.2.1.1) is a key enzyme of the non-oxidizing branch of the pentose phosphate pathway of carbohydrate transformation. Together with transaldolase, it provides the connection between the pentose phosphate pathway and glycolysis [1] and catalyzes the transfer of a two-carbon fragment (glycolaldehyde residue) from ketoses to aldoses. Typical donor substrates of TK are xylulose-5-phosphate (X5P), fructose-5-phosphate, sedoheptulose-5-phosphate, and erythrulose; acceptor substrates are ribose-5-phosphate (R5P), erythrose-4-phosphate, and glyceraldehyde-3-phosphate [2]. General properties of the donor substrates are the following: the presence of a keto group adjacent to the cleavable C–C bond, the presence of a hydroxyl group at the first carbon atom, and *trans*-orientation of the hydroxyl groups at the third and fourth asymmetric carbon atoms. Oxypyruvate and dioxyacetone also can serve as the donor substrates, although they have no asymmetric carbon atoms. The transketolase reaction is reversible except for the case when the substrate is oxypyruvate, which is cleft by the enzyme yielding CO_2.

Transketolase isolated from *Saccharomyces cerevisiae* was the first thiamine diphosphate-dependent enzyme investigated by X-ray analysis [3, 4]. The enzyme is a homodimer of 148.4 kDa [5] with two structurally identical active sites. Each subunits of TK consists of three domains: N, or PP-domain (residues 3-322), medium, or Pyr-domain (residues 323-538), and C-domain (residues 539-680). The first two domains take part in binding of the coenzyme (thiamine diphosphate, TDP). The function of C-domain is still unclear. The coenzyme is located in a deep cleft between the contacting surfaces of the subunits, so that only the C2 atom of the thiazol ring (the binding site of the donor substrate in the catalysis) is accessible to the solvent [3, 4].

Transketolase requires bivalent cations that are necessary for the binding of the pyrophosphate residue of TDP to the apoenzyme. Native holoTK contains only Ca^{2+} (2 g-atom per mol protein, or 1 g-atom per active site) [6]. The data of X-ray analysis indicate that the structures of the apo- and holoTK differ in the spatial orientation of two loops that are localized in different

Abbreviations: DTT, dithiothreitol; GAPD, glyceraldehyde-3-phosphate dehydrogenase; R5P, ribose-5-phosphate; TDP, thiamine diphosphate; TK, transketolase; X5P, xylulose-5-phosphate.

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monomers of the TK dimer. The loops (residues 187-198 and 383-394, respectively) are relatively flexible in apoTK and structured in holoTK [7].

Besides Ca^{2+}, other bivalent cations (for example Mg^{2+}) can serve as the cofactor of TK. Replacement of one cation by another significantly influences the properties of the enzyme. In the presence of Ca^{2+}, the affinity of TDP to apoTK is significantly higher than in the presence of Mg^{2+} [8-10]. Other properties also change, which is indicated by data obtained with the His103 mutant of TK. The mutation did not affect TDP binding to the apoenzyme in the presence of Ca^{2+}, but significantly influenced the process if Mg^{2+} was used instead of Ca^{2+} [11]. These results indicate that the structures of the TK active sites formed in the presence of Ca^{2+} or Mg^{2+} are different. This is supported by other experimental results [12].

The goal of the work was to investigate the interaction of the substrates with two active sites of TK and their catalytic activity.

MATERIALS AND METHODS

Chemicals. The following chemicals were used: TDP, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), NAD^+, R5P, glycylglycine, and $CaCl_2$ (MP Biomedicals, Germany); sodium arsenate and dithiothreitol (DTT) (Fluka, Switzerland); Sephadex G-50 (Pharmacia, Sweden).

Isolation of transketolase. ApoTK was isolated from baker's yeast as described earlier [13] and stored at 4°C in ammonium sulfate of 50% saturation, pH 7.6. Before experiments, the enzyme was desalted on a Sephadex G-50 column. The enzyme was homogenous by the data of SDS-PAGE and exhibited specific activity of 20 U/mg. The concentration of TK was determined spectrophotometrically using $A^{1\%}_{1cm} = 14.5$ at 280 nm [14].

Determination of K_m for R5P and X5P. K_m values for R5P and X5P were determined by curve fitting of the experimental dependence of the reaction rate on substrate concentration. The catalytic activity of TK was measured spectrophotometrically (Aminco DW 2000, Japan) by the rate of NAD^+ reduction in the presence of GAPDH as the coupling enzyme [2]. The reaction mixture (final volume 1 ml) contained 50 mM glycylglycine-HCl, pH 7.6, 2.5 mM $CaCl_2$ ($MgCl_2$), 1 mM sodium arsenate, 3.2 mM DTT, 0.74 mM NAD^+, 0.1 mM TDP, 4 U of GAPDH, 1.5 mM R5P (0.9 mM X5P), and different concentrations of X5P (R5P). The reaction was started by the addition of TK. The data for holoTK (the enzyme with two functioning active sites) were analyzed using the following reaction scheme:

$$\begin{array}{ccc} & K_{m1} & K_{m2} \\ E & \leftrightarrow & ES_1 \leftrightarrow ES_2, \\ & \downarrow V_1 & \downarrow V_2 \end{array}$$

where E is the enzyme, S is the substrate, V_1 and V_2 are the maximal rates of the reaction, and K_{m1} and K_{m2} are the Michaelis constants for the dimer with two bound substrate molecules. The corresponding equation for the reaction rate is the following:

$$\upsilon = \frac{V_2 + V_1 K_{m2}/[S]}{1 + K_{m2}/[S] + K_{m1}K_{m2}/[S]^2} \quad . \qquad (1)$$

In the case when only one of two active sites functioned (semi-holoTK1 and semi-holoTK2), the data were analyzed using the Michaelis equation:

$$\upsilon = V[S]/([S] + K_m). \qquad (2)$$

Preparation of semi-holoTK1. Semi-holoTK1 is the enzyme with only one functioning active site. Originally, the active sites of TK are structurally identical. In the presence of Ca^{2+} they become nonequivalent: the affinity of the coenzyme to one of them (conventionally, active site 1) is rather high (K_d = 32 nM), which is approximately 10-fold higher than the affinity of TDP to the other active site (conventionally, active site 2) [8]. After addition of an equimolar concentration of TDP to 3.3-13.5 μM apoTK solution, the coenzyme completely binds to active site 1 and does not dissociate during the experiment. The semi-holoTK1 was reconstituted from apoTK and TDP (3.3 μM each) in 50 mM glycylglycine buffer, pH 7.6, in the presence of 2.5 mM $CaCl_2$ for 30 min at 25°C. During the experiments on determination of K_m(R5P) and K_m(X5P) values for semi-holoTK1, no TDP was added to the reaction mixture.

Preparation of semi-holoTK2. Semi-holoTK2 is the enzyme where only the active site with low affinity to the coenzyme is functioning. To obtain such a preparation, it is necessary to block active site 1 with high affinity to TDP using an inactive analog of the coenzyme, oxythiamine diphosphate (its affinity to TK active sites is several times higher than the affinity of native TDP) [15]. Then the TK with blocked active site 1 was supplemented with TDP at a concentration that was sufficient to bind to active site 2, but insufficient to displace the oxyTDP from active site 1.

Semi-holoTK2 was prepared in 50 mM glycylglycine buffer, pH 7.6, in the presence of Ca^{2+}: apoTK (3.3 μM) was incubated for 30 min at 25°C in the presence of 3.3 μM oxyTDP, and then TDP was added to the final concentration of 100 μM. The preparation of semi-holoTK2 was used to determine the K_m values for R5P and X5P.

Preparation of X5P and oxythiamine diphosphate. Sodium salt of X5P was synthesized enzymatically by a method developed in our laboratory using oxypyruvate and glyceraldehyde-3-phosphate as the substrates and TK from bakers yeast as the enzyme [16]. The TDP analog (oxyTDP) was synthesized as described earlier [17].

Determination of TDP and oxyTDP. Concentrations of TDP and oxyTDP were determined spectrophotometrically by absorption, using molar absorption coefficient 7500 $M^{-1}\cdot cm^{-1}$ at 272.5 nm for TDP and 8600 $M^{-1}\cdot cm^{-1}$ at 272 nm for oxyTDP [17].

Determination of X5P and R5P. Concentration of X5P was determined spectrophotometrically from the amount of NADH formed during the oxidation of glyceraldehyde-3-phosphate (the product of X5P cleavage by TK). R5P served as the second substrate, and GAPDH was used as the coupling enzyme. The reaction mixture (2 ml) contained 50 mM glycylglycine, 10 mM sodium arsenate, 3.2 mM DTT, 0.37 mM NAD^+, 4 U of GAPDH, 1 mM R5P, 15-20 μg/ml of holoTK, pH 7.6, 25°C. The reaction was started by the addition of 0.05-0.2 μmol of X5P, and the mixture was incubated until X5P was completely exhausted. The reaction was monitored by the change in the absorption of the reaction mixture at 340 nm. The amount of X5P was calculated using the molar absorption coefficient for NADH of 6220 $M^{-1}\cdot cm^{-1}$.

Concentration of R5P was determined in the same way, using 1 mM X5P and starting the reaction by the addition of 0.05-0.2 μmol of R5P. The reaction mixture was incubated until the complete exhaustion of R5P.

RESULTS AND DISCUSSION

Effect of X5P concentration on TK activity. The effect of the donor substrate (X5P) on the catalytic activity of holoTK in the presence of Ca^{2+} at constant concentration of the acceptor substrate (R5P) cannot be described by Michaelis–Menten kinetics, this suggesting nonequivalence of the TK active sites towards X5P. In fact, the experimental points can be adequately fitted to the curve obtained using Eq. (1) (Fig. 1), where the K_m value for active site 2 (K_{m2}) exceeded the K_{m1} value more than 20-fold (table).

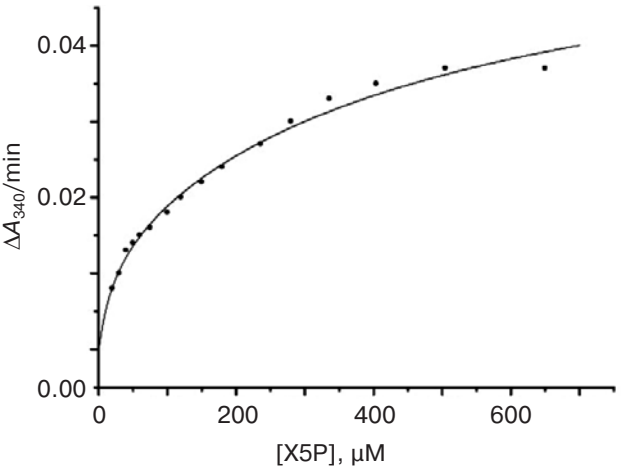

Fig. 1. Effect of X5P concentration on activity of holoTK in the presence of Ca^{2+}: the reaction mixture contained 1.5 mM R5P and 5-650 μM X5P.

The experimental data obtained with semi-holoTK1 (as well as with semi-holoTK2) are reasonably well described by the Michaelis–Menten equation (Fig. 2).

The active sites of TK demonstrate nonequivalence not only in substrate binding, but also in the rate of the catalyzed reaction, which is almost 4-fold higher for active site 2 than for active site 1 (table). However, the activity of semi-holoTK1 does not differ from the activity of semi-holoTK2 (table). The presented data indicate that in the presence of Ca^{2+}, the enzyme exhibits strong negative active site cooperativity in X5P binding and positive cooperativity in the catalytic activity.

Replacement of the cofactor Ca^{2+} by Mg^{2+} significantly changes the kinetic characteristics of TK. In the

Kinetic parameters determined for different TK forms with different substrates and metal cofactors

Enzyme form	Varied substrate	Cofactor	K_{m1}, μM	K_{m2}, μM	V_1, U/mg	V_2, U/mg
HoloTK	X5P	Ca^{2+}	21 ± 6	500 ± 300	10 ± 4	39 ± 9
Semi-holoTK1	–"–	Ca^{2+}	115 ± 7	115 ± 7	13 ± 0.4	13 ± 0.4
Semi-holoTK2	–"–	Ca^{2+}	38 ± 3	38 ± 3	11 ± 0.4	11 ± 0.4
HoloTK	–"–	Mg^{2+}	71 ± 5	71 ± 5	38 ± 1	38 ± 1
–"–	R5P	Ca^{2+}	14 ± 10	600 ± 300	4 ± 2	44 ± 10
Semi-holoTK1	–"–	Ca^{2+}	120 ± 20	120 ± 20	16 ± 1	16 ± 1
Semi-holoTK2	–"–	Ca^{2+}	82 ± 8	82 ± 8	13 ± 0.5	13 ± 0.5
HoloTK	–"–	Mg^{2+}	400 ± 80	400 ± 80	35 ± 2	35 ± 2

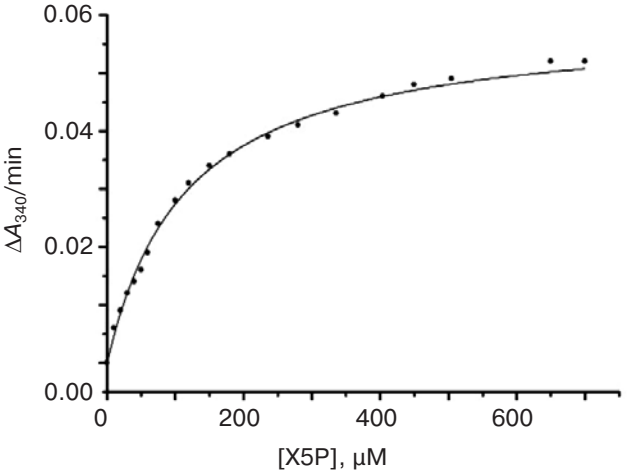

Fig. 2. Effect of X5P on activity of semi-holoTK1 in the presence of Ca^{2+}. Transketolase activity was determined without addition of TDP in the presence of 1.5 mM R5P and 5-700 μM X5P. Experimental data (points) were fitted to Eq. (2) (curve).

presence of Mg^{2+}, the nonequivalence of the active sites in substrate binding disappears: only one K_m value is determined, and the active sites do not differ in their catalytic activity (table). It should be noted that the activity in the presence of Mg^{2+} does not differ from the activity of active site 2 within holoTK in the presence of Ca^{2+} (table).

Effect of R5P concentration on activity of TK. In the presence of Ca^{2+}, the data obtained with R5P differ little from the data obtained with X5P. The K_{m2} value is significantly (more than 40-fold) higher than K_{m1}, and the activity of active site 2 exceeds the activity of active site 1 more than 10-fold (table). The values of V and K_m for R5P for individually functioning active sites in semi-holoTK1 and semi-holoTK2 are virtually the same, taking into account the measurements error, or differ only slightly (table).

In the presence of Mg^{2+}, only one K_m value is determined for both X5P and R5P, which points to the active site equivalence in binding of the substrates, and one value for the rate of the transketolase reaction. The affinity of R5P to the enzyme is lower compared to X5P (table).

Thus, in the presence of Ca^{2+} the active sites of TK exhibit negative cooperativity in binding both X5P (donor substrate) and R5P (acceptor substrate) and positive cooperativity in their catalytic transformation. In the presence of Mg^{2+} instead of Ca^{2+}, the nonequivalence is not exhibited.

This work was supported by the Russian Foundation for Basic Research (grant 06-04-48395).

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