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Interaction of Transketolase from Human Tissues with Substrates

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Abstract—The Michaelis constant values for substrates of transketolase from human tissues were determined over a wide range of substrate concentrations. It is shown that K_m values determined by other authors are significantly overestimated and explained why this is so.

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Key words: human tissues transketolase, thiamine diphosphate, K_m for substrates of human tissues transketolase

Transketolase (TK; EC 2.2.1.1) is a key enzyme of the non-oxidative branch of the pentose-phosphate pathway of conversion of carbohydrates and is a thiamine diphosphate (TDP)-dependent enzyme. TK is detected in all the studied organs and tissues of plants and animals and also in microorganisms [1-4]. TK is a transferase, demonstrates wide substrate specificity, and catalyzes reversible transfer of a two-carbon fragment from keto-substrates to aldo-substrates [1]. TK has been isolated from many sources including human tissues. TK is a homodimer and uses bivalent metal cations and TDP as cofactors.

Saccharomyces cerevisiae transketolase (sTK) is the most studied. In 1992 it was the first TDP-dependent enzyme whose structure was determined by X-ray analysis [5]. There are X-ray structural data for apo- and holoenzyme [6, 7], for TK complexes with TDP analogs [8, 9], and with acceptor substrate [10]. Now sTK can be considered as a model enzyme for development of methods for study of properties of TK from various sources.

Recently human TK (hTK) has attracted considerable interest [11, 12] because its functioning is somehow related with development of Wernicke–Korsakoff syndrome, alcoholism, Alzheimer's disease, and oncological diseases [13, 14]. In particular, enhanced expression of a TK-like protein (TKTL1) in human tumor cells was shown [15-17], this protein differing from TK of normal tissues in primary structure. Human TK has higher substrate specificity than the well-studied sTK. It uses only phosphorylated ketoses (xylulose-5-phosphate (X-5-P), fructose-6-phosphate (F-6-P), sedoheptulose-7-phosphate) as donor substrates and phosphorylated aldoses (ribose-5-phosphate (R-5-P), 3-phosphoglycerol aldehyde) and free glycolic aldehyde as acceptor substrates. Unlike all other studied TKs, hTK is inactive against hydroxypyruvate [4].

In fact, the study of hTK properties has only begun. Of the enzyme properties described in literature we have chosen K_m for substrates: reported values are significantly higher than those determined by us [18]. Those given in [19] seem to be also significantly overestimated.

The goal of this work is to determine why K_m values for hTK substrates determined by various authors differ so much.

MATERIALS AND METHODS

Reagents. The following reagents were used in this work: NAD^+, glyceraldehyde-3-phosphate dehydrogenase (GAPD) from rabbit muscle, TDP, $MgCl_2$, R-5-P, F-6-P, glycyl glycine, and sodium arsenate from Sigma-

Abbreviations: DOETDP, α,β-dioxyethylthiamine diphosphate; DTT, dithiothreitol; F-6-P, fructose-6-phosphate; GAPD, glyceraldehyde-3-phosphate dehydrogenase; hTK, human transketolase; R-5-P, ribose-5-phosphate; sTK, *Saccharomyces cerevisiae* transketolase; TDP, thiamine diphosphate; TK, transketolase; X-5-P, xylulose-5-phosphate.

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Aldrich Chemie GmbH (Germany), dithiothreitol (DTT) from Fluka (Germany). X-5-P was synthesized as described earlier [20] and its barium salt thus obtained had 88% purity and did not contain aldehyde impurities.

Preparation of transketolase. Recombinant hTK was obtained as the holoenzyme by heterolytic expression in *Escherichia coli* as described earlier [18]. Its specific activity estimated by method 1 (see below) was 3 U/mg. According to SDS-PAGE data, the preparation was homogenous. The concentration of hTK was determined using its molar extinction coefficient at 280 nm, which was calculated via amino acid sequence using the ProtParam package [21]; it appeared to be 0.8 $(mg \cdot ml)^{-1} \cdot cm^{-1}$ for the holoenzyme containing two TDP molecules.

Activity estimation. Transketolase activity was estimated via the rate of NAD^+ reduction using GAPD as an auxiliary enzyme and X-5-P and R-5-P as substrates [2] (method 1) or via the rate of oxidation of the α-carbanionic intermediate formed as a result of F-6-P cleavage [22] (method 2):

$$\text{DOETDP} + 2Fe(CN)_6^{3-} + H_2O \xrightarrow{TK} \text{TDP} +$$

$$+ CH_2OH{-}COOH + 2Fe(CN)_6^{4-} + 2H^+.$$

For method 1, the reaction mixture contained in final volume 1 ml: 50 mM glycyl glycine, 10 mM sodium arsenate, 0.37 mM NAD^+, 3 U GAPD, 3.2 mM DTT, 2.5 mM $MgCl_2$, 0.2 mM TDP, 0.05-0.25 μg hTK, 0.25-0.50 mM X-5-P, 1.0 mM R-5-P, pH 7.6. X-5-P and R-5-P concentrations varied on K_m determination are given in figures and the table. Reaction was initiated by addition of X-5-P. Spectra were recorded in 1-cm pathlength cuvettes at 340 nm using an AMINCO DW 2000 spectrophotometer (USA).

Method 2 is based on decrease in optical absorption at 420 nm caused by reduction of hexacyanoferrate (III) anion. The initial reaction rate was constant during the first 30 sec. The reaction mixture contained in final volume 2 ml: 50 mM glycyl glycine, 2.5 mM $MgCl_2$, 0.1 mM TDP, 1.25 mM $K_3[Fe(CN)_6]$, 152-1100 μM F-6-P, 0.9 mg hTK, pH 7.6. Reaction was initiated by addition of the enzyme. There was no enzyme in the reference cuvette.

X-5-P concentration was determined enzymatically directly before the experiment using yeast TK in a GAPD-conjugated system. The reaction mixture contained 50 mM glycyl glycine, 0.37 mM NAD^+, 10 mM sodium arsenate, 3.2 mM DTT, 2.5 mM $CaCl_2$, 0.2 mM TDP, 1 mM R-5-P, and 11.5 μg/ml sTK, pH 7.6. The reaction was initiated by addition of X-5-P in such amount that optical absorption changed by 0.01-0.05 optical unit during full exhaustion of the substrate. Spectra were recorded in 1-cm pathlength cuvettes at 340 nm in the final volume 2 ml.

RESULTS AND DISCUSSION

Determination of Michaelis constant for X-5-P and R-5-P. The data for hTK affinity to donor substrate (X-5-P) as well as to acceptor substrate (R-5-P) can be found in the literature, but they are significantly different. For example, earlier we found that K_m is 11 and 63 μM for X-5-P and R-5-P, respectively [18], whereas the corresponding values in [4, 11, 23-26] are 270-490 μM for X-5-P and 390-630 μM for R-5-P. Such high K_m values for hTK substrates are also reported in [19] (255 μM for X-5-P and 480 μM for R-5-P).

According to the "ping-pong" kinetic mechanism, substrates (donor and acceptor) are bound in the TK active site in strict consequence. The donor substrate is the first to interact with the enzyme; its cleavage gives the first reaction product – aldose. Then the acceptor substrate is bound; it interacts with DOETDP (α,β-dioxyethylthiamine diphosphate), the transketolase reaction intermediate, and this results in the second reaction product – ketose. For this mechanism the following dependence of the enzymatic reaction rate on concentrations of substrates is observed:

$$v = \frac{V_{max}[A]_0[B]_0}{[B]_0K_A + [A]_0K_B + [A]_0[B]_0}, \quad (1)$$

where V_{max} is the maximal reaction rate at the saturating concentration of both substrates, K_A is the Michaelis constant for donor substrate, K_B is the Michaelis constant for acceptor substrate, $[A]_0$ and $[B]_0$ are the initial concentrations of donor and acceptor substrates, respectively. The data can be analyzed using the Michaelis–Menten equation if one of substrates is in excess. This was precisely so when K_m was determined for substrates in hTK-catalyzed reaction by us [18] and other authors [4, 11, 23-26].

However, it should be taken into account that in the case of TK both substrates (donor and acceptor) have one and the same contact area – the positively charged amino acid residues near the entry to the substrate channel, which interact with the phosphate group of substrates [10, 19]. For yeast TK it was shown that phosphorylated donor substrates and acceptor substrates compete for the binding site in the active center [27]. This suggests that there may also be a competition in the case of human TK. High K_m values obtained by other authors can then be rationalized by this competition, because these values were determined at very high concentrations of one substrate at varied concentration the other: 2 mM X-5-P, 2 and 10 mM R-5-P [4, 11, 19, 23, 26]. In our experiments K_m was determined at fixed substrate concentrations of 175 μM and 1 mM, respectively [18].

That is why we decided to consider this problem; for this at various constant concentrations of one substrate we determined the apparent Michaelis constants ($K_{m\ app}$)

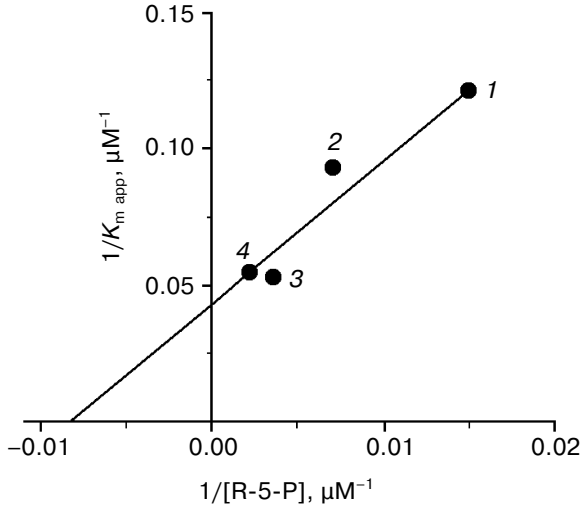

Fig. 1. Reciprocal $K_{m\ app}$ for X-5-P versus reciprocal R-5-P concentration. X-5-P concentration was varied from 8 to 220 μM. R-5-P constant concentration (μM): *1*) 66.3; *2*) 140.6; *3*) 278.5; *4*) 460.

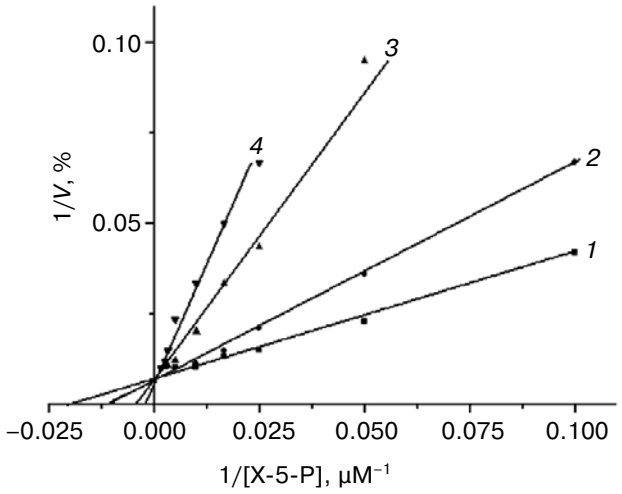

Fig. 2. Activity of hTK versus X-5-P concentration at high R-5-P concentrations. X-5-P concentration was varied from 10 to 600 μM. R-5-P constant concentrations (mM): *1*) 1; *2*) 2; *3*) 5; *4*) 10.

for the other substrate. According to Eq. (1), $K_{m\ app}$ reciprocal values linearly depend on reciprocal concentrations of the first substrate. The intersection of this line with the X-axis gives the negative reciprocal K_m value for the first substrate, and intersection with the Y-axis reveals the K_m for the second substrate.

The reciprocal $K_{m\ app}$ for X-5-P versus reciprocal R-5-P concentration is presented in Fig. 1. The intersection of this line with the X-axis gives K_m = 116 μM for R-5-P, and the intersection with the Y-axis gives K_m = 22 μM for X-5-P. Both values are close to those obtained by us earlier (63 and 11 μM, respectively [18]) and are significantly lower than those obtained by other authors.

The data obtained using high concentrations of the fixed substrate (R-5-P) indicate that our suggestion about competition between substrates as the origin of high K_m values (literature data) is true. All the curves of TK reaction rate versus X-5-P concentration in double-reciprocal coordinates intersect in one point on the Y-axis (Fig. 2), which contradicts Eq. (1) and indicates that X-5-P and R-5-P compete for the binding site of the enzyme. The K_m values for X-5-P in the presence of various R-5-P concentrations including those used in the cited literature are given in the table. As can be seen, on increased R-5-P concentration K_m for X-5-P significantly increases, and at 10 mM R-5-P (the maximal concentration of the fixed substrate used by several authors on determination of K_m for X-5-P) K_m attains the maximal value comparable with those obtained earlier (270-490 μM) [4, 11, 23].

K_m for X-5-P at various R-5-P concentrations (according to Hanes [28])

[R-5-P], mM	K_m for X-5-P, μM
1	40 ± 5
2	100 ± 10
5	200 ± 20
10	410 ± 30

The K_m values of hTK for X-5-P and R-5-P obtained by us seem to be more reasonable than those from literature also in the physiological aspect. As is known, concentrations of TK substrates including X-5-P and R-5-P are 1-100 μM in tissues [4], whereas K_m values for these substrates given in literature are several times higher (270-490 and 390-630 μM, respectively). It is unlikely that hTK can function under such conditions *in vivo*, whereas K_m values obtained by us (11-40 and 63-116 μM) fit better to concentrations of substrates in human tissues.

Determination of K_m for F-6-P. The rate of the transketolase reaction at various F-6-P concentrations was determined via the rate of oxidation of the α-carbanionic intermediate (DOETDP) with hexacyanoferrate (III) anion. Substrate concentration was varied from 152 to 1100 μM. The K_m (340 μM) was determined using the Michaelis–Menten equation. It should be noted that F-6-P affinity to hTK appeared to be significantly lower than X-5-P affinity (at the minimal saturating concentration of R-5-P co-substrate). This may be significant under physiological conditions. The matter is that F-6-P is a typical substrate for glycolysis, and X-5-P and TK are, respectively, a substrate and key enzyme of the pentose-phosphate pathway for conversion of carbohydrates. That is why when phosphorylated saccharides compete for the active site of the enzyme (as mentioned above), X-5-P

having significantly lower K_m for hTK than F-6-P has an obvious advantage.

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