

Research Article

# The mechanism of a one-substrate transketolase reaction

 Olga N. Solovjeva<sup>1</sup>, Marina V. Kovina<sup>2</sup>, Maria G. Zavialova<sup>3</sup>, Victor G. Zgoda<sup>3</sup>, Dmitrii S. Shcherbinin<sup>3</sup> and German A. Kochetov<sup>1</sup>

<sup>1</sup>Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University, 119992 Moscow, Russian Federation; <sup>2</sup>Advanced Cell Technologies Department, Sechenov First Moscow State Medical University (Sechenov University), Trubetskaya Street 8, 119991 Moscow, Russian Federation; <sup>3</sup>Institute of Biomedical Chemistry, Pogodinskaya 10, 119121 Moscow, Russian Federation

**Correspondence:** German A. Kochetov (kochetov@belozersky.msu.ru)



Transketolase catalyzes the transfer of a glycolaldehyde residue from ketose (the donor substrate) to aldose (the acceptor substrate). In the absence of aldose, transketolase catalyzes a one-substrate reaction that involves only ketose. The mechanism of this reaction is unknown. Here, we show that hydroxypyruvate serves as a substrate for the one-substrate reaction and, as well as with the xylulose-5-phosphate, the reaction product is erythrulose rather than glycolaldehyde. The amount of erythrulose released into the medium is equimolar to a double amount of the transformed substrate. This could only be the case if the glycol aldehyde formed by conversion of the first ketose molecule (the product of the first half reaction) remains bound to the enzyme, waiting for condensation with the second molecule of glycol aldehyde. Using mass spectrometry of catalytic intermediates and their subsequent fragmentation, we show here that interaction of the holotransketolase with hydroxypyruvate results in the equiprobable binding of the active glycolaldehyde to the thiazole ring of thiamine diphosphate and to the amino group of its aminopyrimidine ring. We also show that these two loci can accommodate simultaneously two glycolaldehyde molecules. It explains well their condensation without release into the medium, which we have shown earlier.

## Introduction

Transketolase (TK; EC 2.2.1.1), a typical representative of the thiamine diphosphate-dependent enzymes, requires for its activity thiamine diphosphate as a coenzyme as well as divalent cations:  $Mg^{2+}$ ,  $Mn^{2+}$ , or  $Ca^{2+}$  that ensure binding between the coenzyme and the apoprotein [1,2]. TK is widespread in nature and has been found in all studied organs and tissues of animals, plants and also in microorganisms [3–5].

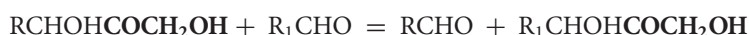
TK is involved in the production of precursors of many vitally important substances in the living cell, nucleotides, coenzymes, aromatic amino acids, and others [6].

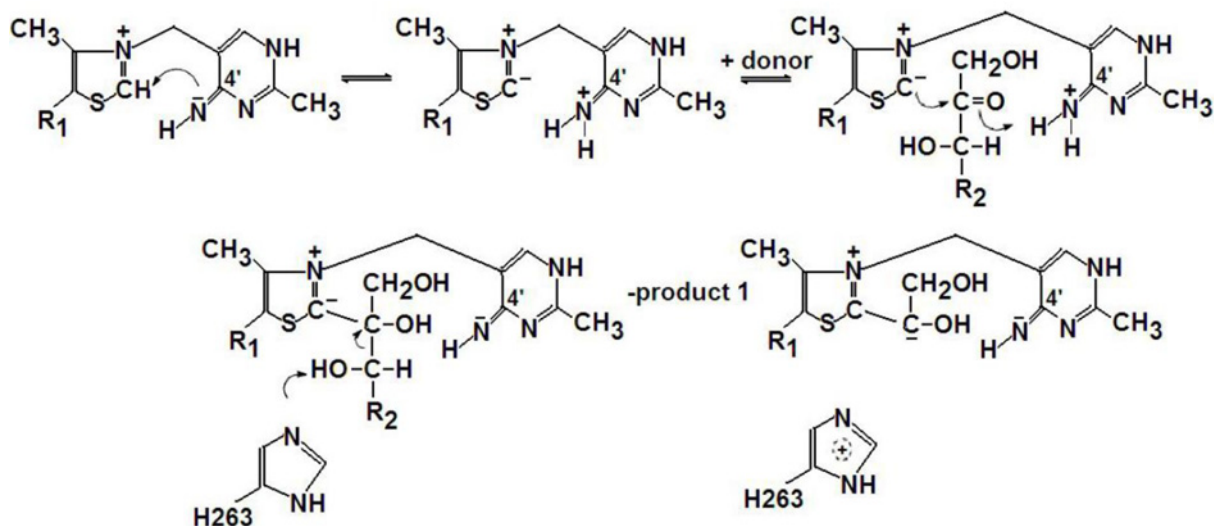
Together with transaldolase, TK is involved in the interaction between glycolysis and the pentose phosphate pathway of carbohydrate metabolism [7], which enables the cell to become adapted to different metabolic requirements.

Transketolase catalyzes the reversible cleavage of the C2–C3 bond in keto sugars (donor substrates  $RCHOHCOCH_2OH$ ) [3,4,8]. The cleaved-off two-carbon fragment of the keto substrate (the active glycolaldehyde) becomes covalently bound to ThDP, namely (as is currently believed) to its thiazole ring and then, without being released into the medium, is transferred to the aldo sugar (the acceptor substrate  $R_1CHO$ ). The rest of the donor substrate is released into the medium as the product aldo sugar  $RCHO$  [9].

Received: 28 January 2018  
Revised: 28 January 2018  
Accepted: 27 February 2018

Version of Record published:  
04 August 2020





**Scheme 1.** The classical ThDP activation mechanism

(According to Shnider and Lindquist 1998 [9]).

In the absence of an aldo sugar a one-substrate reaction takes place – only the donor substrate is utilized [10,11].



The mechanism of this reaction remained unknown, here we aim to understand it. The first half of this reaction is predictable, as we know that the ThDP activation mechanism includes two closely linked processes [9]: deprotonation of the C2 thiazole ring (the site of the subsequent keto substrate binding) and stabilization of the resulting carbanion by the aminopyridine moiety (Scheme 1).

As follows from Scheme 1, the classical mechanism of the two-substrate transketolase reaction assumes the binding of the first substrate (ketose) and the glycol aldehyde residue (GliA) which is cleaved from it, to the C2 of the thiazole ring. Therefore, it is presently accepted that the second substrate (aldose) becomes bound to the GliA residue rather than to C2. However, in the one-substrate reaction the second substrate is also ketose, and another GliA residue is cleaved off from the second ketose molecule and condenses with the first one [10,11]. It seems logical to suppose therefore that the second ketose molecule, like the first one, might be bound to and cleaved at the C2 of thiazole, whereas the first GliA residue moves over to an alternative binding site. If an alternative binding site exists, a few options are possible *a priori*:

1. The first GliA moiety is bound to the thiazole ring of ThDP, and the second substrate molecule binds to the pre-attached GliA (this option coincides with the presently accepted mechanism of the two-substrate transketolase reaction).
2. The first GliA moiety is bound to the thiazole ring, while the second substrate molecule binds to another site, for example, to the aminopyrimidine ring or to the protein.
3. The first GliA moiety binds to (is transferred to) another binding site, for example, aminopyrimidine ring via a Schiff base, after which the second keto substrate molecule binds to the thiazole ring and is cleaved producing the second GliA molecule. Subsequently, the two GliA molecules undergo condensation forming erythrulose at the initial or another binding site.

In the present study we employed mass-spectrometry and 3D modeling to determine which of the above-mentioned options is correct.

## Experimental

The following chemicals were used:  $\alpha$ -glyceraldehyde 3-phosphate dehydrogenase from rabbit muscle,  $\text{NAD}^+$ , ThDP, glycylglycine,  $\text{CaCl}_2$ , formic acid (Sigma), dithiothreitol, sodium arsenate (Fluka, Germany), Sephadex G-50 (Pharmacia, Sweden). Other reactants were of extra pure grade.

## Transketolase purification

Transketolase was isolated from baker's yeast *Saccharomyces cerevisiae* as described earlier [12], aliquoted and stored frozen in 20 mM potassium phosphate buffer. The enzyme was homogeneous by SDS-PAGE and exhibited a specific activity of 30 U/mg. The concentration of TK was determined spectrophotometrically using  $A^{1\%}_{1\text{cm}} = 14.5$  at 280 nm [13]. Prior to use, the TK solution was passed through a Sephadex G-50 column, equilibrated with 50 mM glycylglycine, pH 7.6.

## Preparation of holotransketolase

TK at 10–15 mg/ml was supplemented with 2.5 mM  $\text{CaCl}_2$  and ThDP, whose concentration was twice the molar concentration of TK (i.e. one ThDP for each active center).

## Preparation of the phosphopentose mixture

The barium salt of the phosphopentose mixture containing xylulose 5-phosphate and ribose 5-phosphate was prepared using an earlier described method [14]. The barium salt was converted to the potassium salt before use.

## Measurement of transketolase activity

The catalytic activity of TK was measured using spectrophotometry by the rate of  $\text{NAD}^+$  reduction in a coupled system with glyceraldehyde 3-phosphate dehydrogenase [15]. The reaction mixture in the final volume of 1 ml contained 50 mM glycylglycine, 2.5 mM  $\text{CaCl}_2$ , 1 mM sodium arsenate, 3.2 mM dithiothreitol, 0.1 mM ThDP, 1.6 mM  $\text{NAD}^+$ , 3 units of glyceraldehyde 3-phosphate dehydrogenase, and 3.2 mg/ml potassium salt of a phosphopentose mixture, which was used as the substrate, at pH 7.6. The reaction was initiated by the addition of the phosphopentose solution.

## Determination of thiamine diphosphate

The concentration of ThDP was determined spectrophotometrically by measuring the optical density at 272.5 nm (using the molar extinction coefficient  $7500 \text{ M}^{-1} \text{ cm}^{-1}$ ) [16].

## Erythrulose determination

Erythrulose accumulation was performed in the following reaction mixture: 50 mM glycylglycine, 10 mg/ml TK, 0.1 mM  $\text{CaCl}_2$ , 0.17 mM ThDP, 1 mM hydroxypyruvate (HPA), pH 7.6. After the total depletion of HPA in the one-substrate reaction (about 4 h), the reaction mixture was treated with 10% chloric acid, spinned off from the protein precipitate, and neutralized with KOH. Erythrulose was determined by the method described earlier [17].

## ESI-MS and MS/MS analysis

The samples were in 40 mM ammonia, 40 mM formic acid and 5% TCA, pH 4.0, dissolved in water.

We analyzed samples using an LTQ Orbitrap mass spectrometer. A sample was injected at 3  $\mu\text{l}/\text{min}$  with nebulizer gas flow of 8 l/min. The temperature of the inlet capillary was  $270^\circ\text{C}$ , and the capillary voltage, 3.6 kV. The mass spectra of positive ions were measured using a FTMS analyzer with mass resolution of 60,000 in the mass range of 200–1000 Da. The mass accuracy was above 5 ppm. We used tandem mass-spectra to clarify the composition and structure of the analyte. The appropriate ions were isolated in a 2 Da mass window and subjected to collision-induced dissociation (CID). The resulting spectra were obtained by averaging 300 scans.

## Molecular 3D modeling

The spatial structures of TK with covalently bound GliA to ThDP were obtained from the Protein Data Bank (PDB) (PDBid 1GPU) [18]. Preliminary optimization of the structures was performed using the SYBYL 8.1 software package, which was also used for the additional modification of ThDP in which the second GliA was bound to the pyrimidine ring. The structures were minimized in vacuum using the Tripos force field and Gasteiger–Huckel atomic partial charges.

We have designed a preliminary model of the cofactor with two GliA residues bound to TK by adding a second GliA to the cofactor with covalently bound GliA (PDB id 1GPU), then we optimized the model by minimization and molecular dynamics.

Molecular dynamics simulations were performed using the Amber8 software package. Parameterization was carried out using the ff99-SB force field for protein molecules and the GAFF force field for cofactor atoms.

The TIP3P water model was used for solvation and  $\text{Na}^+$  ions were added for system neutralization.

**Table 1 Consumption of HPA and formation of erythulose in a one-substrate reaction catalyzed by TK**

Initial HPA load, mM	Amount of erythulose formed		
	Theoretically expected, mM	Actually formed, mM	% of the expected
1.0	0.5	0.48	96
0.5	0.25	0.23	92

Erythulose was determined according to [17] after the total depletion of HPA and subsequent protein removal.

At the first stage sequential minimization of the system was performed in vacuum and in a solvent (for 25,000 steps). The next stage included sequential heating of the system (up to 300 K) and pressure increase (up to 1 atm) during 40 ps using NVT and NPT ensembles. Particle mesh Ewald (PME) was employed to treat the long-range electrostatic interactions and the cut-off for the non-bonded interactions was set to 8 Å. The temperature was maintained using Langevin dynamics with a friction coefficient of 2 ps<sup>-1</sup>, and pressure was controlled with a Berendsen barostat. Molecular dynamics was performed on 10 ns trajectories with 2 fs time steps.

MD results were analyzed using the AMBER program tool ptraj and the VMD software [19].

## Results

### Identification and quantization of the one-substrate reaction product

In this study, we employed HPA as the substrate of the one-substrate transketolase reaction. Its product erythulose was determined as equimolar to the initial HPA load divided by a factor two (Table 1). Therefore, this reaction is similar to that with xylulose-5-phosphate [10,11]. The reaction with HPA is irreversible since one of its products is CO<sub>2</sub>, and for this reason we expect that a greater proportion of ThDP should be bound to the reaction's intermediate than in the reversible reaction with xylulose-5-phosphate.

### Preparation of samples for mass-spectrometry

The TK holoenzyme (holoTK) was incubated with HPA for 4 h at room temperature in the presence or absence of NaCNBH<sub>3</sub>.

Following incubation the sample was passed through a Sephadex G-50 column equilibrated with 30 mM NH<sub>4</sub>OH and 40 mM formic acid, pH 6.5. The fractions containing protein with bound ThDP and ThDP derivatives (the intermediates) were collected. The protein was then denatured with 5% TCA and removed by centrifugation. The supernatant was used for mass spectrometry.

### Mass spectrometry of intermediates formed in the one-substrate transketolase reaction

Figure 1 shows mass spectra of ThDP intermediates formed in the first half of the one-substrate reaction, the binding and cleavage of the first HPA molecule as a substrate, in the absence and presence of NaCNBH<sub>3</sub> (spectra 2 and 3, respectively) together with the control mass spectrum of the holoTK (spectrum 1).

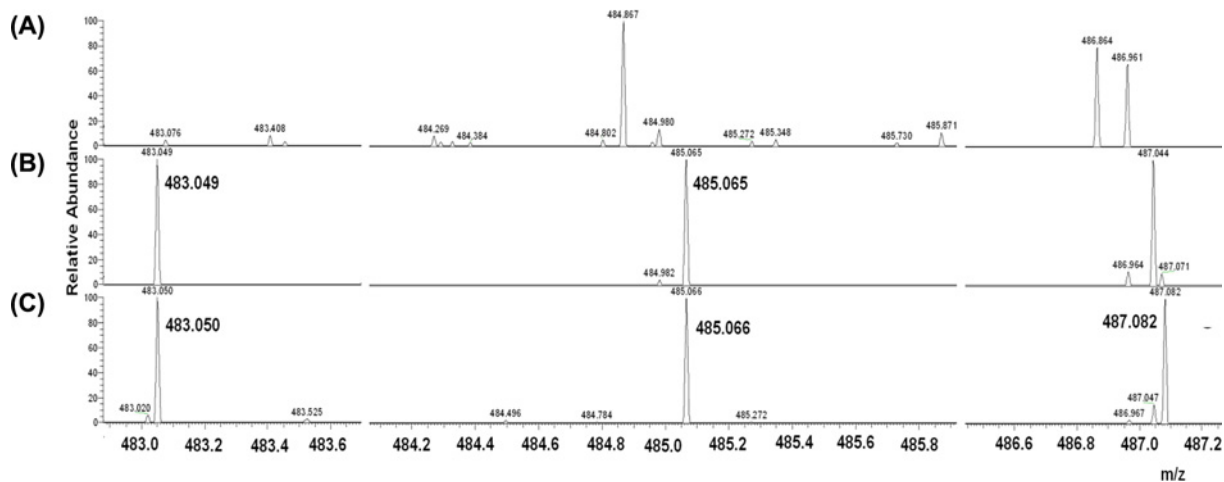
Mass spectra in the wider range (from 480 to 550 Da) are given in Supplemental Figure S1.

Both in the presence and absence of NaCNBH<sub>3</sub>, the mass spectra of the first semi-reaction intermediates (spectra 3 and 2, Figure 1) reveal the appearance of 1<sup>+</sup> charged molecular ions with *m/z* (mass) of 483.050 and 485.066, corresponding to the calculated sums of masses of GliA and ThDP (dehydroThDP): 60.0211 + 425.0449 (423.0292) = 485.0660 (483.0503).

The mass 487.082 appears only in the spectra of intermediates produced in the presence of NaCNBH<sub>3</sub> (Figure 1, spectrum 3) and corresponds to the dihydride of the compound with mass 485: 485.066 + 2\*(1.0078) = 487.082.

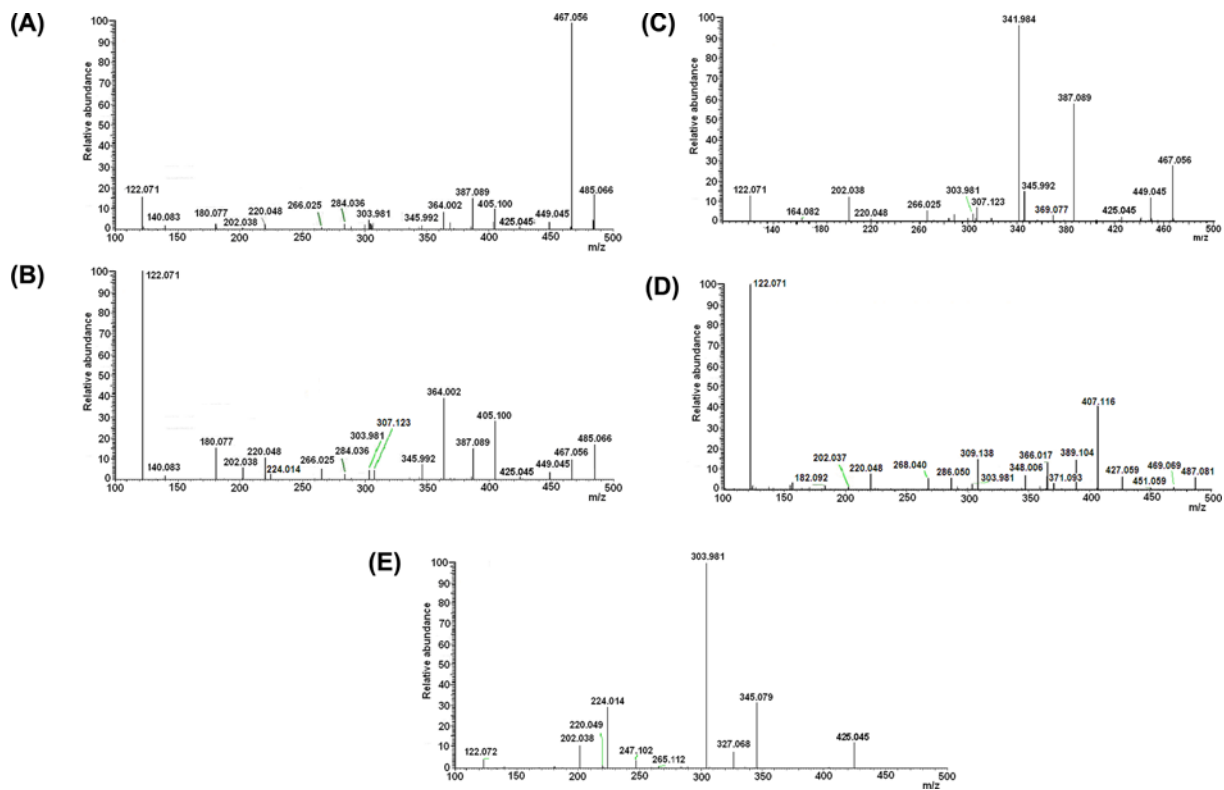
None of the expected masses, 483.050, 485.066, 487.082, appears on trace 1 of Figure 1, the mass spectrum of holoTK, while it has a few contaminant peaks (484.867, 486.961). The approach to refine spectra from contaminant peaks is discussed in the Supplementary Material.

Let us now consider what information on the structure of these intermediates can be provided by their fragmentation.



**Figure 1.** ESI-MS spectra of holoTK reaction medium in the range from 482.9 to 487.3 Da

(A) ESI-MS spectra of holoTK in the absence of HPA and NaCNBH<sub>3</sub>; (B) in the presence of HPA; (C) in the presence of HPA and NaCNBH<sub>3</sub>.

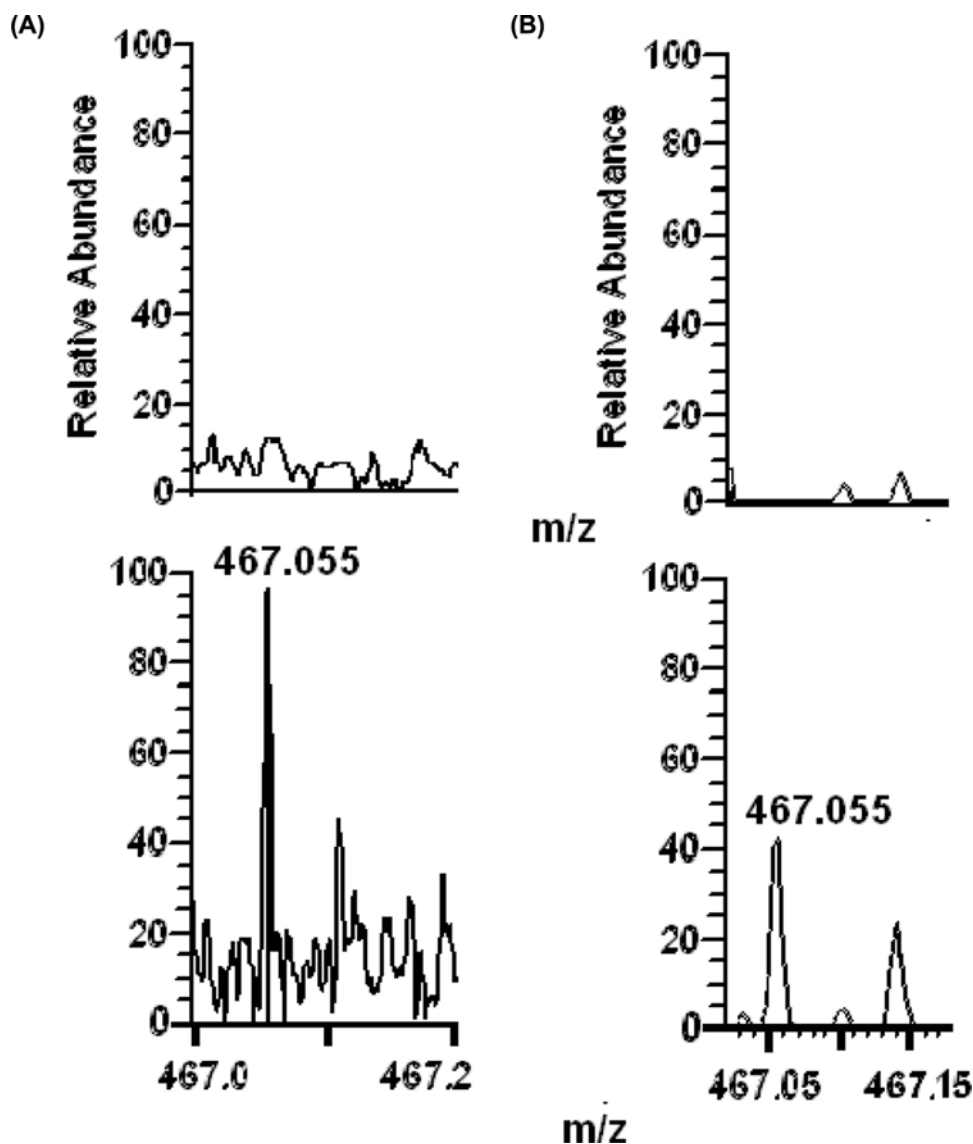


**Figure 2.** Typical ESI-MS/MS spectra of isolated intermediates of holoTK reaction with HPA

(A and B): mass 485.066 Da; (C): mass 467.056 Da; (D): mass 487.081 Da; (E): mass 425.045 Da. Mass 425.045 was obtained from ESI-MS spectra of the TK holoenzyme in the absence of HPA and NaCNBH<sub>3</sub>.

## Fragmentation of intermediates of the one-substrate transketolase reaction

The main fragment of intermediate 485.066 subjected to mild fragmentation is its dehydration product with the experimentally obtained mass of 467.056, in a good agreement with the calculated value:  $485.066 - 2 \times (1.0078) - 15.995 = 467.0557$  (Figure 2A).



**Figure 3.** ESI-MS spectra of holoTK reaction medium in the range from 467 to 467.2 Da

The mixtures of intermediates are produced by holoTK with HPA (lower graphs) or without HPA (upper graphs) in the absence (A) or presence (B) of NaCNBH<sub>3</sub>.

Under more rigid fragmentation conditions, the amplitude of mass 467.056 decreases while amplitudes of smaller masses increase, including mass 180.076, with mass values remaining unchanged (compare Figure 2B and Figure 2A). The peak 467.056 is also present in the main mass spectra (Figure 3A,B).

Fragments of mass 467.056 of the main mass spectrum are present in the fragmentation set of mass 485.066 (compare Figure 2C and Figure 2B), with exception of masses corresponding to dehydration or deoxygenation (decreased by 18.011 or 15.994):

$$\begin{aligned}
 449.045 &= 467.056 - 18.011 \\
 387.089 &= 405.100 - 18.011 \\
 345.992 &= 364.002 - 18.010 \\
 164.082 &= 180.076 - 15.994 \\
 122.071 &= 140.081 - 18.010
 \end{aligned}$$

Thus, the peak 467.056 of the main spectrum is formed by the dehydrated intermediate 485.066, whose dehydration may occur under non-fragmenting conditions either spontaneously or enzymatically.

**Table 2 MS/MS fragments of masses 425.045, 485.066, 487.082, and 467.056**

Molecular formula	Calculated masses	Experimental fragments of the mass ( <i>m/z</i> )			
		425	485	487	467
C <sub>14</sub> O <sub>9</sub> H <sub>23</sub> S <sub>1</sub> N <sub>4</sub> P <sub>2</sub> *	485.066	-	485.066	-	-
C <sub>14</sub> O <sub>9</sub> H <sub>25</sub> S <sub>1</sub> N <sub>4</sub> P <sub>2</sub> *	487.082	-	-	487.081	-
C <sub>14</sub> O <sub>8</sub> H <sub>21</sub> S <sub>1</sub> N <sub>4</sub> P <sub>2</sub> *	467.056	-	467.056	467.055	467.055
C <sub>14</sub> O <sub>8</sub> H <sub>23</sub> S <sub>1</sub> N <sub>4</sub> P <sub>2</sub> *	469.071	-	-	469.069	-
C <sub>14</sub> O <sub>7</sub> H <sub>19</sub> S <sub>1</sub> N <sub>4</sub> P <sub>2</sub> *	449.045	-	449.045	-	449.043
C <sub>14</sub> O <sub>7</sub> H <sub>21</sub> S <sub>1</sub> N <sub>4</sub> P <sub>2</sub> *	451.061	-	-	451.059	-
C <sub>12</sub> O <sub>7</sub> H <sub>19</sub> S <sub>1</sub> N <sub>4</sub> P <sub>2</sub>	425.045	425.045	425.045	-	425.044
C <sub>12</sub> O <sub>7</sub> H <sub>21</sub> S <sub>1</sub> N <sub>4</sub> P <sub>2</sub> *	427.061	-	-	427.059	-
C <sub>14</sub> O <sub>6</sub> H <sub>22</sub> S <sub>1</sub> N <sub>4</sub> P <sub>1</sub> *	405.100	-	405.100	-	-
C <sub>14</sub> O <sub>6</sub> H <sub>24</sub> S <sub>1</sub> N <sub>4</sub> P <sub>1</sub> *	407.116	-	-	407.116	-
C <sub>14</sub> O <sub>5</sub> H <sub>20</sub> S <sub>1</sub> N <sub>4</sub> P <sub>1</sub> *	387.089	-	387.089	-	387.089
C <sub>14</sub> O <sub>5</sub> H <sub>22</sub> S <sub>1</sub> N <sub>4</sub> P <sub>1</sub> *	389.105	-	-	389.105	-
C <sub>8</sub> O <sub>9</sub> H <sub>16</sub> S <sub>1</sub> N <sub>1</sub> P <sub>2</sub> *	364.002	-	364.002	-	-
C <sub>8</sub> O <sub>9</sub> H <sub>18</sub> S <sub>1</sub> N <sub>1</sub> P <sub>2</sub> *	366.018	-	-	366.017	-
C <sub>12</sub> O <sub>4</sub> H <sub>18</sub> S <sub>1</sub> N <sub>4</sub> P <sub>1</sub>	345.079	345.079	-	-	-
C <sub>8</sub> O <sub>8</sub> H <sub>14</sub> S <sub>1</sub> N <sub>1</sub> P <sub>2</sub> *	345.992	-	345.992	-	345.991
C <sub>8</sub> O <sub>8</sub> H <sub>16</sub> S <sub>1</sub> N <sub>1</sub> P <sub>2</sub> *	348.006	-	-	348.006	-
C <sub>12</sub> O <sub>3</sub> H <sub>16</sub> S <sub>1</sub> N <sub>4</sub> P <sub>1</sub>	327.068	327.068	-	-	-
C <sub>8</sub> O <sub>7</sub> H <sub>12</sub> S <sub>1</sub> N <sub>2</sub> P <sub>2</sub> *	341.984	-	-	-	341.983
C <sub>14</sub> O <sub>2</sub> H <sub>19</sub> S <sub>1</sub> N <sub>4</sub> *	307.123	-	307.123	-	307.122
C <sub>14</sub> O <sub>2</sub> H <sub>21</sub> S <sub>1</sub> N <sub>4</sub> *	309.138	-	-	-	-
C <sub>6</sub> O <sub>7</sub> H <sub>12</sub> S <sub>1</sub> N <sub>1</sub> P <sub>2</sub>	303.981	303.981	303.981	303.981	303.980
C <sub>8</sub> O <sub>6</sub> H <sub>15</sub> S <sub>1</sub> N <sub>1</sub> P <sub>1</sub> *	284.036	-	284.0355	284.036	-
C <sub>8</sub> O <sub>6</sub> H <sub>17</sub> S <sub>1</sub> N <sub>1</sub> P <sub>1</sub> *	286.052	-	-	286.050	-
C <sub>8</sub> O <sub>5</sub> H <sub>13</sub> S <sub>1</sub> N <sub>1</sub> P <sub>1</sub> *	266.025	-	266.024	-	266.025
C <sub>8</sub> O <sub>5</sub> H <sub>15</sub> S <sub>1</sub> N <sub>1</sub> P <sub>1</sub> *	268.040	-	-	268.040	-
C <sub>6</sub> O <sub>4</sub> H <sub>11</sub> S <sub>1</sub> N <sub>1</sub> P <sub>1</sub>	224.014	224.014	224.014	224.014	-
C <sub>6</sub> O <sub>4</sub> H <sub>13</sub> S <sub>1</sub> N <sub>1</sub> P <sub>1</sub>	-	-	-	226.029	-
C <sub>6</sub> O <sub>4</sub> H <sub>11</sub> N <sub>3</sub> P <sub>1</sub>	220.049	220.049	220.048	220.048	220.048
C <sub>6</sub> O <sub>3</sub> H <sub>9</sub> N <sub>3</sub> P <sub>1</sub>	202.038	202.038	202.038	202.037	202.038
C <sub>8</sub> O <sub>2</sub> H <sub>10</sub> N <sub>3</sub> *	180.077	-	<b>180.077</b>	<b>180.076</b>	-
C <sub>8</sub> O <sub>2</sub> H <sub>10</sub> N <sub>3</sub> *	182.093	-	-	<b>182.092</b>	-
C <sub>8</sub> O <sub>1</sub> H <sub>10</sub> N <sub>3</sub> *	164.082	-	-	-	<b>164.082</b>
C <sub>6</sub> H <sub>8</sub> N <sub>3</sub>	122.072	122.072	122.071	122.071	122.072

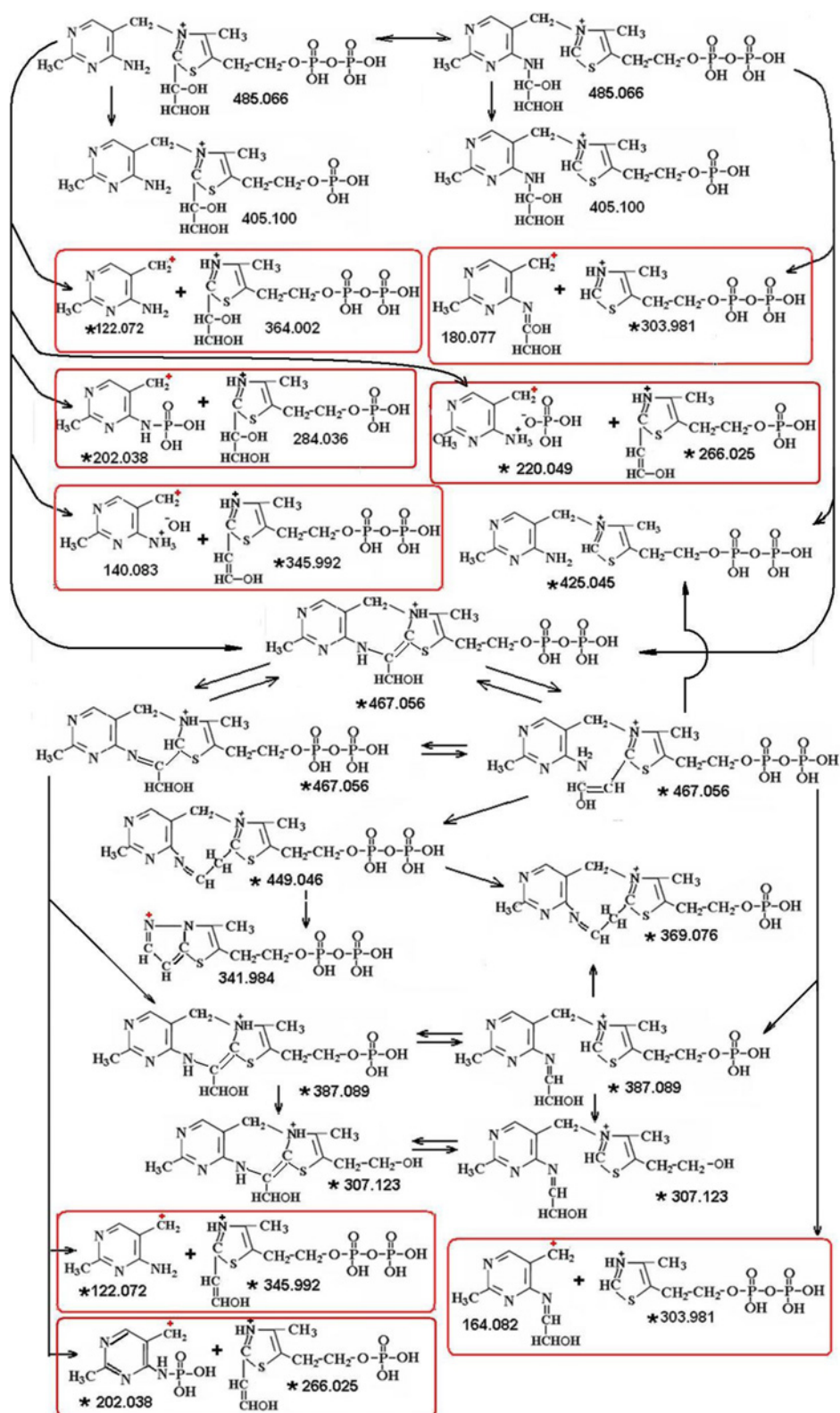
The GliA-containing masses are marked with an asterisk (\*).

Based on the masses we have determined the atomic composition (Table 2) and the sequence of fragmentation of intermediates 485.066 and 467.056 (Scheme 2). It includes:

- (i) cleavage off of water (485.066 - 18.011 = 467.055, 467.056 - 18.011 = 449.045), phosphate (485.066 - 79.966 = 405.100), phosphate and water (485.066 - 18.011 - 79.966 = 387.089), diphosphate and water (485.066 - 18.011 - 159.933 = 307.122),
- (ii) dissociation of the GliA moiety with formation of the initial ThDP (425.045),
- (iii) breakage of the molecule between the rings with or without loss of phosphate or water: 122.072 + 345.992 (+18), 122 + 364, 122.072 + 284.036 (+80), 122.072 + 266.025 (+18+80), 220.049 + 266.025, 202.038 + 284.036 (266+18), **180.077 + 303.981. 164.082+303.981.**

Noteworthy are the two latter combinations given in bold: the masses 180.076 and 164.082 correspond to those of the pyrimidine ring of ThDP with bound GliA or its deoxy-derivative (Scheme 2).

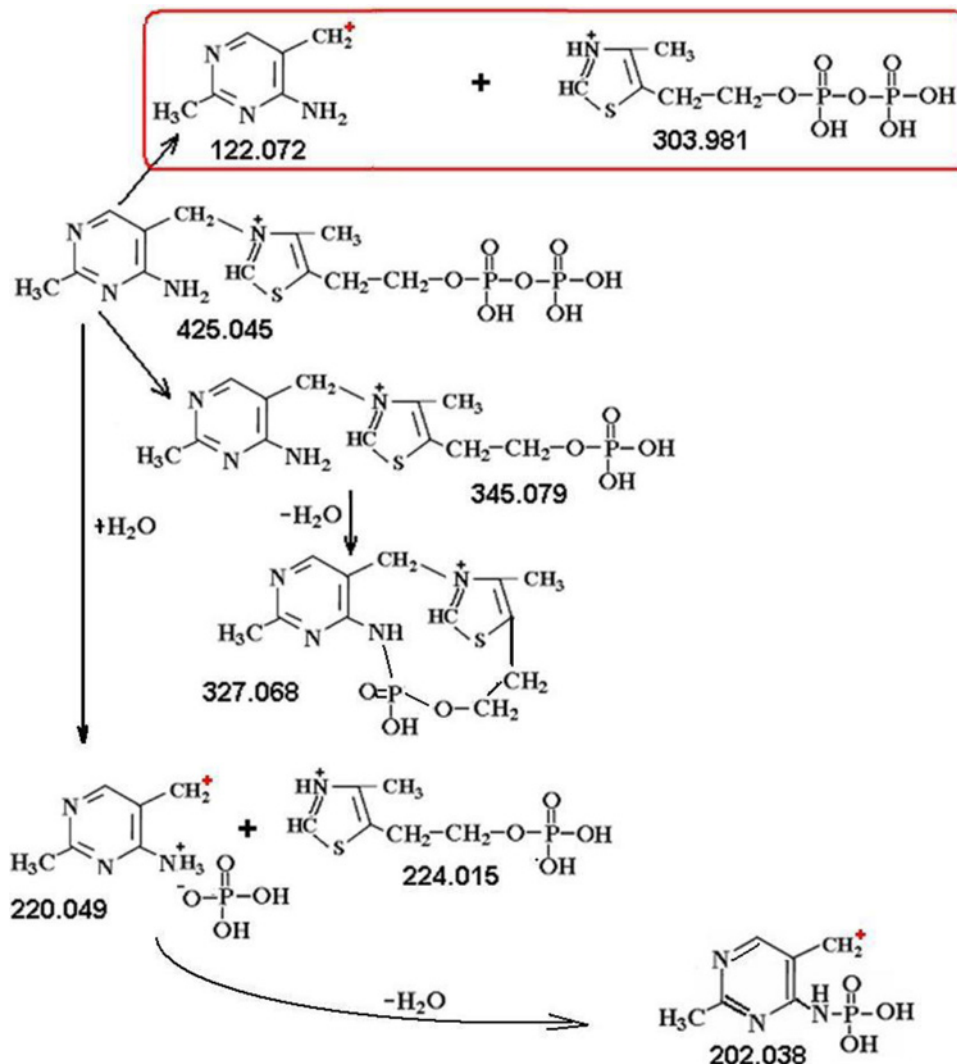
To confirm the proposed structures, we fragmented ThDP as a compound with known structure (Figure 2E and Scheme 3). Majority of its fragmentation masses coincided with GliA-free fragment masses of intermediates:



**Scheme 2. Proposed structures of the intermediate 485.066 Da and its fragments**

The lower part of the scheme shows the family of its main fragment 467.056 Da, which is also present as an independent intermediate in the TK-reaction medium. The framework combines complementary fragments. Repeating masses are marked with an asterisk (\*), all fragments are charged positively (separation at the anode). The positive charge produced when the electron is separated from the radical upon breaking the bond is marked +.





### Scheme 3. Structural formulas of proposed ThDP fragments

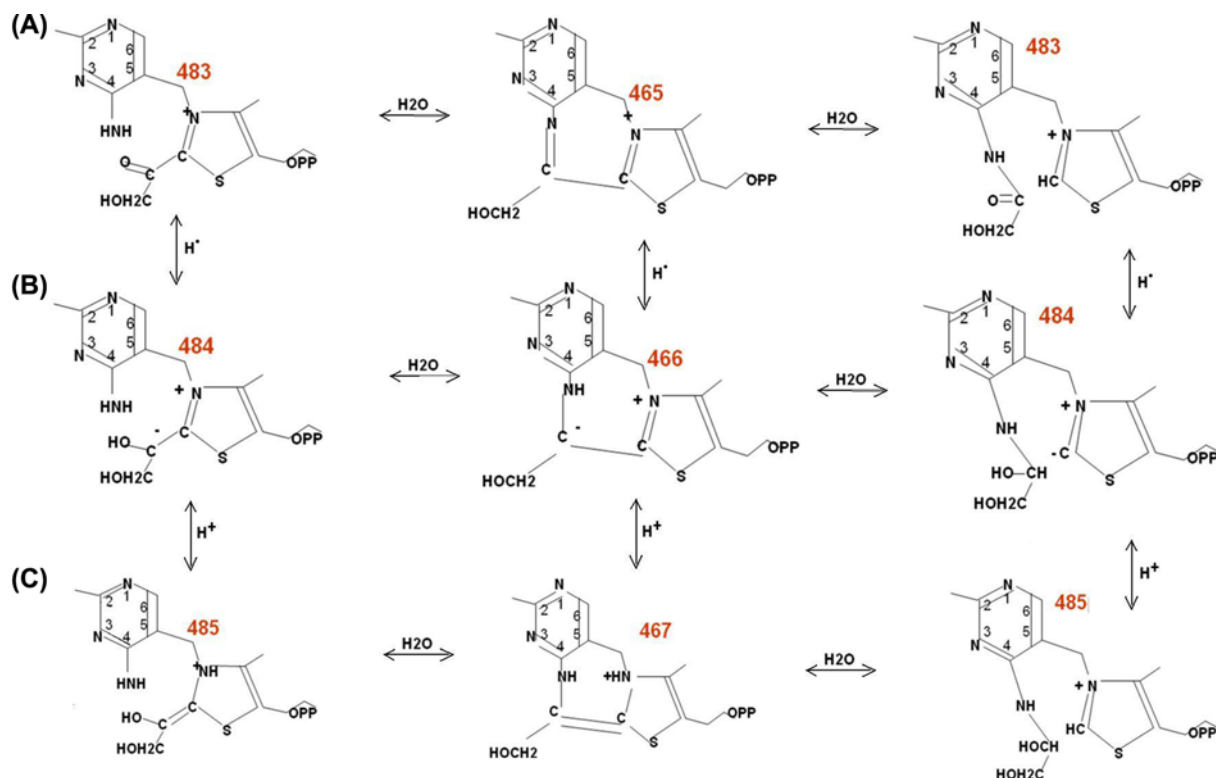
The framework encloses complementary fragments. All fragments are charged positively (separation at the anode). The positive charge produced when the electron is separated from the radical upon breaking the bond is marked +.

dephospho \ dehydrates 345.079, 327.068, 265.112, 247.102 and mono-rings 122.072, 303.981, 224.015, 220.049, 202.038, see Table 2.

In the presence of  $\text{NaCNBH}_3$ , the intermediate 485.066 is hydrogenated to mass 487.082. A comparison of the fragmentation spectra of intermediates 485.066 and 487.082 (Figure 2B,D, Table 2) suggests that hydrogenation does not prevent fragmentation of intermediate 487.082 at the same loci as for intermediate 485.066, but some of the masses, including the important mass 180.077, are increased by  $\sim 2.015$  Da, i.e. by two hydrogen masses:  $180.077 + 2 \times 1.0078 = 182.093$ .

## Discussion

A comparison of the fragmentation spectrum of ThDP as a substance of known structure with that of the intermediates 485, 467, and 487 whose structure we determined by mass spectrometry shows that the fragments identified by us as GliA-containing (marked with an asterisk in Table 2) were present only in the spectra of intermediates and completely absent in the fragmentation spectrum of ThDP, while most GliA-free peaks were observed both in the fragmentation spectra of intermediates and ThDP. Thus, the reliability of determining the structure of fragments by the mass spectrometry method was confirmed not only by the exact agreement between the expected and experimental masses



**Scheme 4. A tricycle as a structure enabling GliA exchange between the ThDP loci**

GliA transition is accompanied with (de)protonation and (de)hydrogenation of the intermediate.

(accurate to the third decimal place) but also by the expected presence/absence of GliA-containing/not-containing masses in the spectra of fragmentations of the intermediates and of the ThDP control.

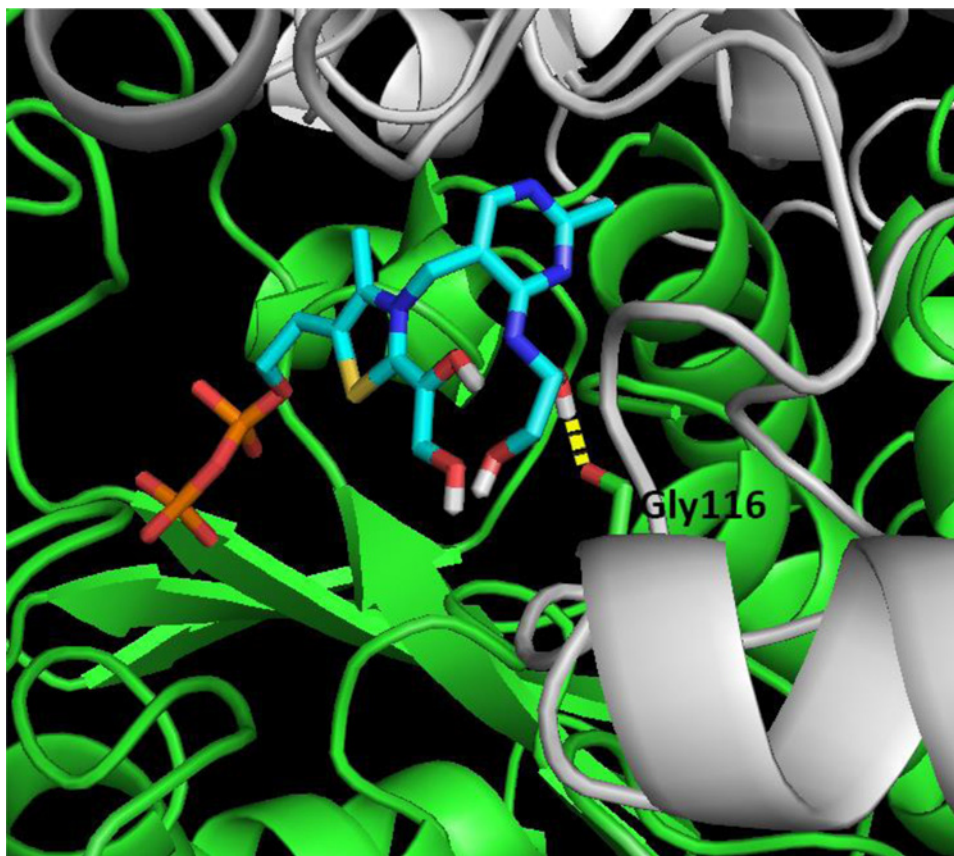
The existence of covalent bonding of GliA with the amino-group of the pyrimidine ring is confirmed by the formation of mass 180.076 upon fragmentation of intermediate 485.050, as well as by the inverse correlation of its amplitude with that of the largest 467-fragment (compare A and B in Figure 2). The mass-identification of this product indicates that it corresponds to the structure of the GliA-adduct of the aminopyrimidine ring of ThDP.

It is interesting to note that the formation of mass 180.076 from the initial mass 485.065 is accompanied by dehydrogenation, so that the total mass of both fragments,  $180.076 + 303.981 (-1.008) = 483.050$ , corresponds to a spontaneously dehydrogenated product 483 of the native protein-bound intermediate  $484^-$ , shown in the Scheme 1. This is indicative of a possible spontaneous inter-conversion between forms 485 and 483, found in the protein-free mass spectra and confirms our assumption about the equally probable and reversible transformation of protein-bound carbanion  $484^-$  into its protein-free derivatives 483 and 485.

The result obtained in the present study, the covalent binding of glycolaldehyde to the aminopyrimidine ring of ThDP, suggests that the amino group and the C2 of the thiazole ring are equally capable of binding the transferred residue of the ketosubstrate, the GliA moiety. Apparently, a binding equilibrium of GliA to these two loci of ThDP is possible through formation of a tricycle accompanied by reversible dissociation of water (Scheme 4).

The existence in the initial mass-spectra of intermediates 483, 485, and the dehydrated 467 offers a good support for the above Scheme. The intermediates 465,  $466^-$ , 467 should have tricyclic structures, since non-cyclic structures of this type are chemically unstable. The possibility of a tricycle formation was shown earlier for both ThDP [20] and its derivatives with aldehydes [21].

The observation of the fragment with mass 164.082 in the fragmentation spectrum of the dehydrated product 467 offers an additional proof for the existence of a covalent bond between GliA and the amino group of ThDP. The mass value (164.082) equals that of aminopyrimidine with deoxo-GliA ( $=180.076 - 15.994$ ), the result of a loss of water during cyclization of intermediate 485 into 467.



**Figure 4. Model of the TK active site with ThDP-GliA intermediate**

Two GliA residues are covalently bound to ThDP via the amino group of the aminopyrimidine ring and the C2 of the thiazole ring. ThDP and GliA atoms are colored as follows: "C" - blue, "N" - dark blue, "P" - orange, "S" - yellow, "O" - red, "H" - white. The enzyme subunits are shown in green. Most of hydrogen atoms of the cofactor are hidden for clarity.

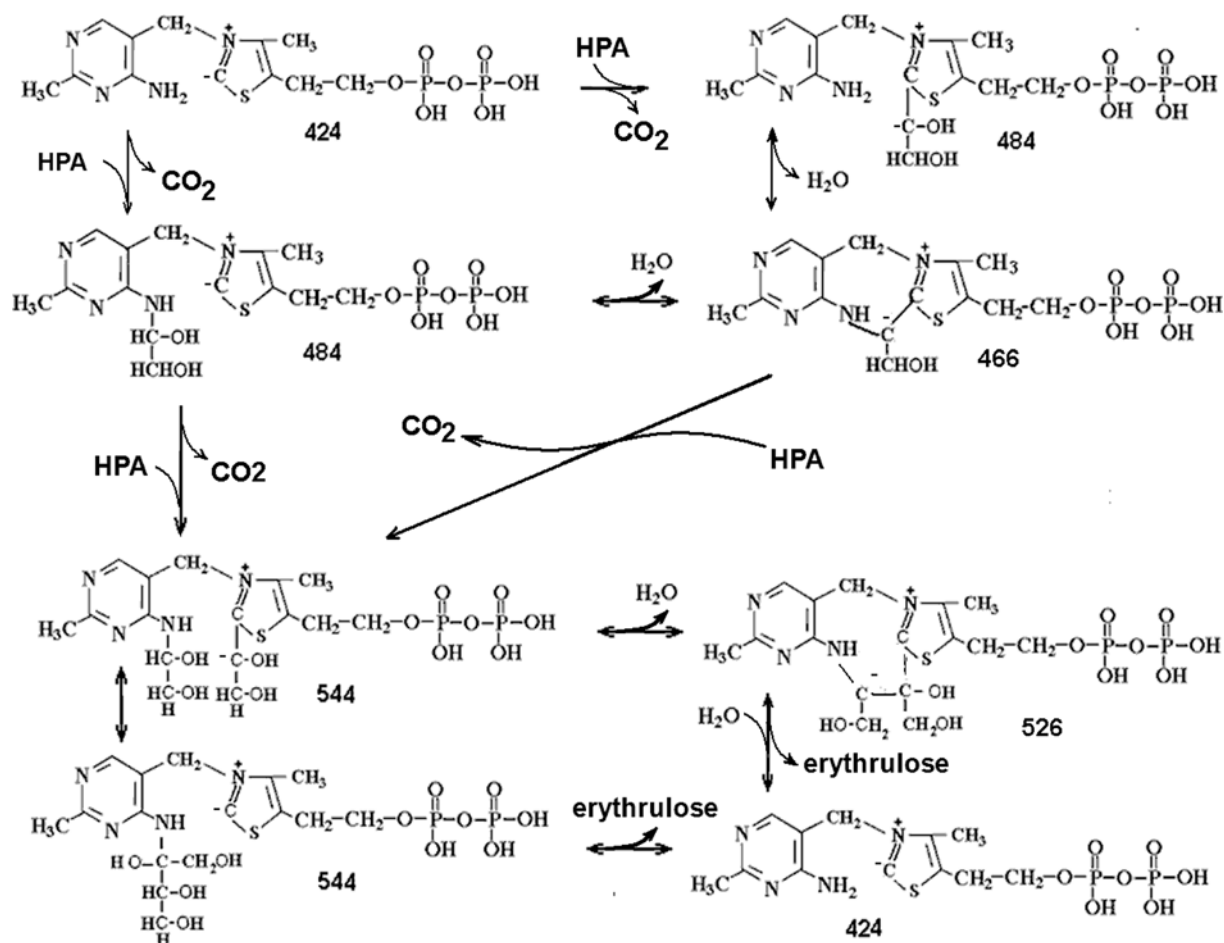
Besides, a small peak 182.092, corresponding to a dihydride of adduct of aminopyrimidine with GliA ( $=180.076 + 2 \times 1.0078$ ), is seen in the fragmentation spectrum of mass 487, a cyanborohydride-reduced derivative of intermediate 485 (Figure 2D).

Therefore, we have found a second binding site for GliA, in addition to thiazolic C2: the aminogroup of pyrimidine. This rules out option 1 (introduction) as a possibility for overall catalysis, namely that the second substrate molecule binds to the first pre-attached GliA.

With the aid of 3D computer modeling we have shown that the holoTK active site can easily accommodate two GliA residues, one at the amino group of the pyrimidine ring and the other, at the C2 site of the thiazole ring of ThDP (Figure 4). The preliminary model of an active site with two GliA residues had small overlap of atomic radii of carboxylic oxygen of glycine-116 and one hydroxyl oxygen of GliA. But molecular dynamics removed the overlap showing that loop Gly116-Pro117-Leu118 is quite flexible.

The 3D modeling has also shown that, the GliA on thiazole would block access from the outside to the amino group thus ruling out option 2 of the initial hypothesis, namely that the second GliA binds on the aminogroup after the first one is bound to thiazole (see 'Introduction' section). Therefore, in view of the presence of the tricycle and the equilibrium between the alternative binding sites for GliA at the different ThDP rings, we conclude that catalysis should require a transfer of the first GliA from thiazole ring and/or its binding to the aminopyrimidine ring prior to the binding of the second substrate molecule on thiazole (Scheme 5). This is option 3 of our initial hypothesis (see introduction).

We had difficulties identifying the four-carbon derivative of ThDP: likely, erythrulose releases to the medium too fast. Another explanation is that ThDP-tetroses appear on our mass spectra as masses 543.055 and 527.060 Da (Figure



**Scheme 5. The proposed scheme of the one-substrate transketolase reaction**

The masses are given for non-charged molecules, differing by 1Da from masses discovered in this study.

1S and Scheme 1S of Supplementary Material) instead of the expected masses 543.072 and 527.077 (=425.044 + 120.0422 - 2 × 1.0077 or -18,01) due to a mass defect. Unstable ions are known to show mass defects [22,23].

In the research presented here we conclude, that HPA, in the same way as shown earlier for xylulose-5-phosphate, serves as a substrate for the one-substrate transketolase reaction, where the erythrose product condenses from two glycolaldehyde residues formed via the decarboxylation of two HPA molecules in the absence of acceptor substrate. At least three independently obtained fragments (164.082, 180.076, and 182.092) of different forms of the main 485-intermediate prove the function of the ThDP amino group as the covalent binding site for the transferred moiety of the substrate. This function of ThDP amino group has never been demonstrated before.

### Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

### Funding

The authors declare that there are no sources of funding to be acknowledged.

### Author Contribution

ON.S: protein isolation and preparation for mass spectrometry; MV.K: the idea and manuscript writing; MG.Z: mass spectrometry; VG.Z: mass spectrometry design and interpretation; D.S: 3D modeling; GA.K: the overall supervision, experimental design, study coordination and data analysis.

All authors contributed into scientific discussions and conclusions

## Acknowledgements

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. Part of this work was carried out using equipment of the Shared-Use Facility Center "Regenerative medicine" of Sechenov University (ID310020).

## Abbreviations

GliA, glycolaldehyde; HPA, hydroxypyruvate; TCA, trichloroacetic acid; ThDP, thiamine diphosphate; TK, transketolase.

## References

- 1 Kochetov, G.A. and Philippov, P.P. (1970) Calcium: cofactor of transketolase from baker's yeast. *Biochem. Biophys. Res. Commun.* **38**, 930–933, [https://doi.org/10.1016/0006-291X\(70\)90810-7](https://doi.org/10.1016/0006-291X(70)90810-7)
- 2 Heinrich, P.C., Steffen, H., Janser, P. and Wiss, O. (1972) Studies on the reconstitution of apotransketolase with thiamine pyrophosphate and analogs of the coenzyme. *Eur. J. Biochem.* **30**, 533–541, <https://doi.org/10.1111/j.1432-1033.1972.tb02124.x>
- 3 Mitschke, L., Parthier, C.H., Schröder-Tittmann, K., Coy, J., Lüdtke, S. and Tittmann, K. (2010) The crystal structure of human transketolase and new insights into its mode of action. *J. Biol. Chem.* **285**, 31559–31570, <https://doi.org/10.1074/jbc.M110.149955>
- 4 Asztalos, P., Parthier, C., Golbik, R., Kleinschmidt, M., Hübner, G., Weiss, M.S. et al. (2007) Strain and near attack conformers in enzymic thiamin catalysis: X-ray crystallographic snapshots of bacterial transketolase in covalent complex with donor ketoses xylulose 5-phosphate and fructose 6-phosphate, and in noncovalent complex with acceptor aldose ribose 5-phosphate. *Biochemistry* **46**, 12037–12052
- 5 Veitch, N.J., Maugeri, D.A., Cazzulo, J.J., Lindqvist, Y. and Barrett, M.P. (2004) Transketolase from *Leishmania mexicana* has a dual subcellular localization. *Biochem. J.* **382**, 759–767, <https://doi.org/10.1042/BJ20040459>
- 6 Kochetov, G.A. and Solovjeva, O.N. (2014) Structure and functioning mechanism of transketolase. *Biochim. Biophys. Acta* **1844**, 1608–1618, <https://doi.org/10.1016/j.bbapap.2014.06.003>
- 7 Hohmann, S. and Meacock, P.A. (1998) Thiamin metabolism and thiamin diphosphate-dependent enzymes in the yeast *Saccharomyces cerevisiae*: genetic regulation. *Biochim. Biophys. Acta* **1385**, 201–219, [https://doi.org/10.1016/S0167-4838\(98\)00069-7](https://doi.org/10.1016/S0167-4838(98)00069-7)
- 8 Sundström, M., Lindqvist, Y. and Schneider, G. (1992) Three-dimensional structure of apotransketolase. Flexible loops at the active site enable cofactor binding. *FEBS Letters* **313**, 229–231, [https://doi.org/10.1016/0014-5793\(92\)81197-T](https://doi.org/10.1016/0014-5793(92)81197-T)
- 9 Schneider, G. and Lindqvist, Y. (1998) Crystallography and mutagenesis of transketolase: mechanistic implications for enzymatic thiamin catalysis. *Biochim. Biophys. Acta* **1385**, 387–398, [https://doi.org/10.1016/S0167-4838\(98\)00082-X](https://doi.org/10.1016/S0167-4838(98)00082-X)
- 10 Bykova, I.A., Solovjeva, O.N., Meshalkina, L.E., Kovina, M.V. and Kochetov, G.A. (2001) One-substrate transketolase-catalyzed reaction. *Biochem. Biophys. Res. Commun.* **280**, 845–847, <https://doi.org/10.1006/bbrc.2000.4199>
- 11 Fiedler, E., Golbik, R., Schneider, G., Tittmann, K., Neef, H., König, S. et al. (2001) Examination of donor substrate conversion in yeast transketolase. *J. Biol. Chem.* **276**, 16051–16058, <https://doi.org/10.1074/jbc.M007936200>
- 12 Solovjeva, O.N. (2002) Isolation and properties of noncovalent complex of transketolase with RNA. *Biochemistry (Moscow)* **67**, 667–671, <https://doi.org/10.1023/A:1016198321838>
- 13 Heinrich, C.P., Noack, K. and Wiss, O. (1972) Chemical modification of tryptophan at the binding site of thiamine-pyrophosphate in transketolase from baker's yeast. *Biochem. Biophys. Res. Commun.* **49**, 1427–1432, [https://doi.org/10.1016/0006-291X\(72\)90498-6](https://doi.org/10.1016/0006-291X(72)90498-6)
- 14 Gubler, C.J., Johnson, L.R. and Wittorf, J.H. (1970) Yeast transketolase (sedoheptulose-7-phosphate:d-glyceraldehyde-3-phosphate dihydroxyacetone transferase, EC 2.2.1.1) assay of thiamine diphosphate. *Methods Enzymol.* **18A**, 120–125, [https://doi.org/10.1016/0076-6879\(71\)18290-0](https://doi.org/10.1016/0076-6879(71)18290-0)
- 15 Kochetov, G.A. (1982) Transketolase from yeast, rat liver, and pig liver. *Methods Enzymol.* **90**, 209–223, [https://doi.org/10.1016/S0076-6879\(82\)90128-8](https://doi.org/10.1016/S0076-6879(82)90128-8)
- 16 Schellenberger, A. and Hübner, G. (1965) On the separation of phosphoric acid esters of thiamine and its analogues by gradient elution. *Hoppe Seylers Z. Physiol. Chem.* **343**, 189–192, <https://doi.org/10.1515/bchm2.1966.343.1.189>
- 17 Morii, K., Hosomi, S., Tomoyuki, T. and Mizoguchi, T. (1985) Methods for enzymatic and colorimetric determinations of D-erythrulose (D-tetrolulose). *Anal. Biochem.* **151**, 188–191, [https://doi.org/10.1016/0003-2697\(85\)90070-3](https://doi.org/10.1016/0003-2697(85)90070-3)
- 18 Fiedler, E., Thorell, S., Sandalova, T., Golbik, R., König, S. and Schneider, G. (2002) Snapshot of a key intermediate in enzymatic thiamin catalysis: crystal structure of the alpha-carbanion of (alpha,beta-dihydroxyethyl)-thiamin diphosphate in the active site of transketolase from *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA* **99**, 591–595, <https://doi.org/10.1073/pnas.022510999>
- 19 Humphrey, W., Dalke, A. and Schulten, K. (1996) VMD – Visual Molecular Dynamics. *J. Mol. Graphics* **14**, 33–38, [https://doi.org/10.1016/0263-7855\(96\)00018-5](https://doi.org/10.1016/0263-7855(96)00018-5)
- 20 Doughty, M.B. and Risinger, G.E. (1985) Studies on the Aminopyrimidinyl Group of Thiamine. *J. Chem. Soc. Chem. Commun.* **22**, 1551–1553, <https://doi.org/10.1039/c39850001551>
- 21 Doughty, M.B., Risinger, G.E. and Jungk, S.J. (1987) Chemistry of the tricyclic form of thiamine with aldehydes in basic ethanol. *Bioorg. Chem.* **15**, 15–30, [https://doi.org/10.1016/0045-2068\(87\)90003-4](https://doi.org/10.1016/0045-2068(87)90003-4)
- 22 Dominic Chan, T.-W., Eva Fung, Y.M. and Leo Li, Y.C. (2002) A Study of Fast and Metastable Dissociations of Adenine-Thymine Binary-Base Oligonucleotides by Using Positive-Ion MALDI-TOF Mass Spectrometry. *J. Am. Soc. Mass. Spectrom.* **13**, 1052–1064, [https://doi.org/10.1016/S1044-0305\(02\)00423-3](https://doi.org/10.1016/S1044-0305(02)00423-3)

- 23 Ruan, Q., Peterman, S., Szewc, M.A., Ma, L., Cui, D., Humphreys, W.G. et al. (2008) An integrated method for metabolite detection and identification using a linear ion trap/Orbitrap mass spectrometer and multiple data processing techniques: application to indinavir metabolite detection. *J. Mass. Spectrom.* **43**, 251–261, <https://doi.org/10.1002/jms.1311>