ISSN 0006-2979, Biochemistry (Moscow), 2009, Vol. 74, No. 3, pp. 293-300. © Pleiades Publishing, Ltd., 2009.
Original Russian Text © L. E. Meshalkina, G. A. Kochetov, G. Hubner, K. Tittmann, R. Golbik, 2009, published in Biokhimiya, 2009, Vol. 74, No. 3, pp. 362-371.
Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM08-238, December 21, 2008.

New Function of the Amino Group of Thiamine Diphosphate in Thiamine Catalysis*

L. E. Meshalkina[1], G. A. Kochetov[1], G. Hubner[2], K. Tittmann[2], and R. Golbik[2]**

[1]*Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University, 119992 Moscow, Russia; fax: (495) 939-3181; E-mail: luda@genebee.msu.ru*
[2]*Martin-Luther-University Halle-Wittenberg, Institute of Biochemistry/Biotechnology, Kurt-Mothes-Strasse 3, Halle/Saale 06120, Germany*

Received July 22, 2008
Revision received August 29, 2008

Abstract—In this work, we investigated the rate of formation of the central intermediate of the transketolase reaction with thiamine diphosphate (ThDP) or 4′-methylamino-ThDP as cofactors and its stability using stopped-flow spectroscopy and circular dichroism (CD) spectroscopy. The intermediates of the transketolase reaction were analyzed by NMR spectroscopy. The kinetic stability of the intermediate was shown to be dependent on the state of the amino group of the coenzyme. The rates of the intermediate formation were the same in the case of the native and methylated ThDP, but the rates of the protonation or oxidation of the complex in the ferricyanide reaction were significantly higher in the complex with methylated ThDP. A new negative band was detected in the CD spectrum of the complex transketolase–4′-methylamino-ThDP corresponding to the protonated dihydroxyethyl-4′-methylamino-ThDP released from the active sites of the enzyme. These data suggest that transketolase in the complex with the NH_2-methylated ThDP exhibits dihydroxyethyl-4′-methylamino-ThDP-synthase activity. Thus, the 4′-amino group of the coenzyme provides kinetic stability of the central intermediate of the transketolase reaction, dihydroxyethyl-ThDP.

DOI: 10.1134/S0006297909030080

Key words: transketolase, thiamine diphosphate, dihydroxyethyl-thiamine diphosphate, 4′-methylamino-thiamine diphosphate, stopped-flow spectroscopy, CD, NMR

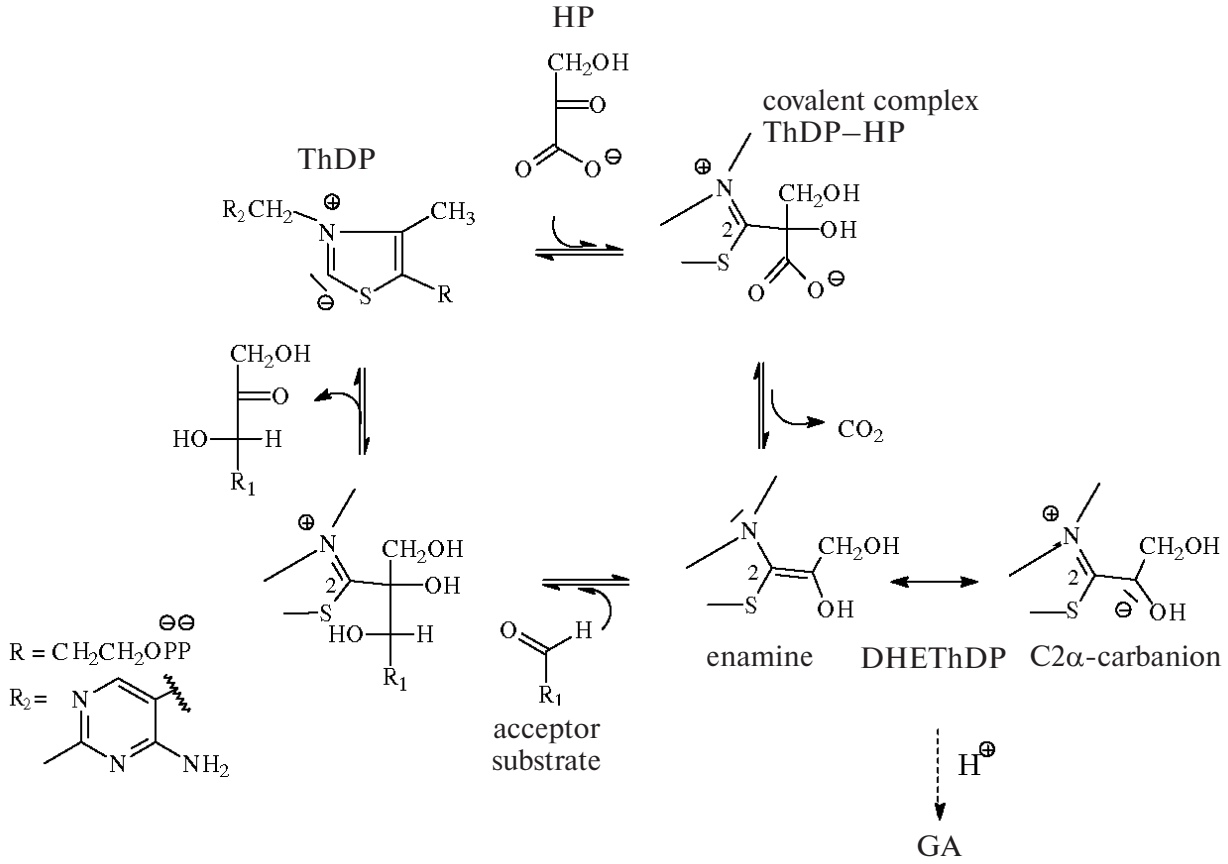
Transketolase (TK, EC 2.2.1.1) is a thiamine diphosphate (ThDP)-dependent enzyme of the pentose phosphate pathway of carbohydrate metabolism. In contrast to other ThDP-dependent enzymes, this enzyme exhibits activity with the methylated analog of its native cofactor (4′-methylamino-ThDP). Previously it was shown that transketolase in complex with the methylated analog of the cofactor is capable of catalyzing the carboligation reaction of β-hydroxypyruvate (donor substrate) and glycolaldehyde (acceptor substrate) yielding erythrulose, as well as the ferricyanide reaction, where the central intermediate of the transketolase reaction (α-carbanion/enamine of dihydroxyethyl-ThDP) oxidizes yielding glycolic acid [1, 2].

Transketolase catalyzes one of the key reactions of the pentose phosphate pathway of carbohydrate metabolism, cleaving ketosugars (donor substrates) at the C–C bond adjacent to the ketogroup with subsequent transfer of the two-carbon fragment to an aldose (acceptor substrate). The cofactors of TK are bivalent metal ions and ThDP. The enzyme exhibits broad substrate specificity. Donor substrates are ketoses containing hydroxyl groups at C3 and C4 positions in the *trans*-conformation: xylulose-5-phosphate, fructose-6-phosphate, sedoheptulose-7-phosphate, erythrulose, etc. Exceptions are three-carbon donor substrates (β-hydroxypyruvate (HP) and dioxyacetone) that have no asymmetric carbon atoms. Acceptor substrates for TK are ribose-5-phosphate, erythrose-4-phosphate, glyceraldehyde phosphate, glycolaldehyde (GA), etc. The enzyme exhibits little specificity

Abbreviations: CD, circular dichroism; DHEThDP, dihydroxyethyl-thiamine diphosphate; GA, glycolaldehyde; GAPD, glyceraldehyde-3-phosphate dehydrogenase; HP, hydroxypyruvate; ThDP, thiamine diphosphate; TK, transketolase.

* This paper is devoted to the 80-th anniversary of the birth of Prof. A. Schellenberger (Martin-Luther-University Halle-Wittenberg, Institute of Biochemistry/Biotechnology, Halle, Germany).

** To whom correspondence should be addressed.



Catalytic cycle of the transketolase reaction with hydroxypyruvate as the donor substrate

Scheme 1

towards the length of the carbon chain, but the presence of the phosphate group in the substrate molecule significantly increases the affinity of the substrate to the enzyme [3]. The reaction catalyzed by TK is reversible except for the case when HP is used as the donor substrate. In this case, HP is subjected to decarboxylation, and the process becomes irreversible.

The TK reaction can be divided into two steps (Scheme 1). The first step includes binding of the donor substrate to ThDP to yield covalent enzyme–substrate complex, cleavage of the donor substrate, and the formation of the central intermediate product, α-carbanion/enamine of dihydroxyethyl-ThDP (DHEThDP), and the first reaction product. In the second step, the formed intermediate interacts with the acceptor substrate yielding the second reaction product, a new ketose. Thus, TK is a typical transferase that requires two substrates: a donor and an acceptor of the two-carbon fragment.

In the absence of the acceptor substrate (one-substrate transketolase reaction), the α-carbanion form of DHEThDP can be protonated at the C2α position with subsequent release of the glycolaldehyde or DHEThDP. Besides, DHEThDP can be oxidized in the presence of a suitable electron acceptor, for example ferricyanide, yielding glycolic acid [3, 4]. It was shown that native TK (in the complex with ThDP) catalyzes the carboligase reaction in the presence of only the donor substrate. The product of this reaction is erythrulose forming as a result of the condensation of two GA residues (the products of the cleavage of two molecules of the donor substrate) [5, 6]. Thus, the presence of the acceptor substrate determines the direction of the transketolase reaction.

It should be noted that the rate of the reactions proceeding in the absence of the acceptor substrate is very low. The intermediate product DHEThDP is stabilized in the active site of the enzyme. Figure 1 demonstrates the net of hydrogen bonds involved in the stabilization of the intermediate and the planar enamine structure with sp^2-hybridization [7]. Based on these data, we assumed that the amino group of ThDP stabilizes the enamine form of the intermediate, while any modification (for example, methylation) causes the opposite effect. To test this assumption, in the present work we used 4′-methylamino-ThDP, a ThDP analog with modified amino group (Scheme 2).

The goal of the investigation was to compare the transketolase reaction with the native coenzyme and with its methylated analog to characterize the rates of forma-

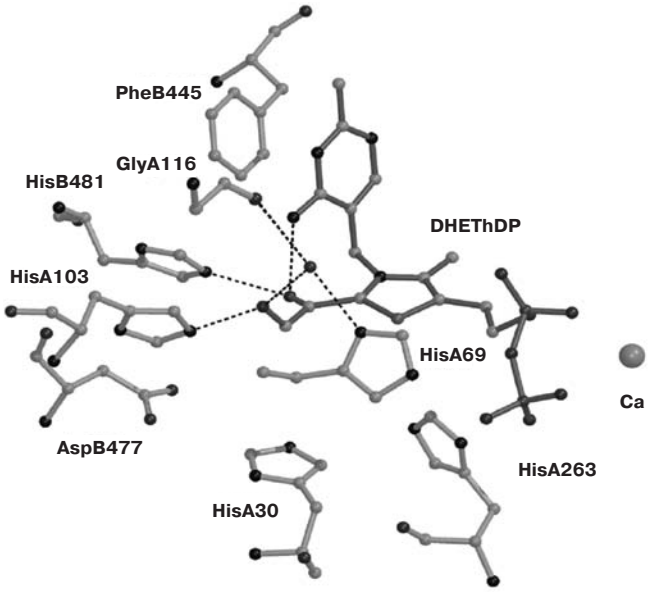
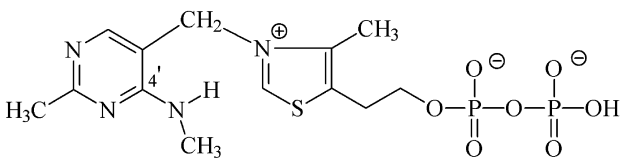


Fig. 1. Environment of the key intermediate of the transketolase reaction DHEThDP in the active site of the enzyme. Hydrogen bonds between the intermediate and the nearby residues (within 3.1 Å) are shown by the dashed line. A and B, relative designation of two different subunits [7].

tion of the key intermediate products DHEThDP and DHE-4′-methylamino-ThDP and their stability. The stability of the intermediates can be estimated by the rate of their oxidation in the presence of ferricyanide and by the rate of their protonation. In both cases, the α-carbanion form of the intermediate takes part in the reaction. An increase in the rate of these reactions while using DHE-4′-methylamino-ThDP as the coenzyme instead of the native coenzyme must indicate a decrease in stability of the enamine form of the intermediate.

MATERIALS AND METHODS

Chemicals. NAD^+, glyceraldehyde-3-phosphate dehydrogenase (GAPD) from rabbit muscles, ThDP, $CaCl_2$, lithium salt of β-hydroxypyruvate, xylulose-5-phosphate, and ribose-5-phosphate were from Sigma–Aldrich Chemie GmbH (Germany), glycolaldehyde from ICN Biomedicals Incorporation (USA), and dithiothreitol and $CaCl_2$ from Fluka (Germany). The 4′-methylamino-ThDP was synthesized as described earlier [8, 9]. The purity of the chemically synthesized analog was tested by ^{1}H-NMR and mass-spectrometric analyses before and after phosphorylation: the absence of ThDP in the preparation was confirmed.



Structure of 4′-methylamino-ThDP

Scheme 2

Isolation of transketolase. Recombinant TK with specific activity of 37 U/mg was isolated as described previously [10] with minor modifications [11]. The enzyme was homogeneous according to the results of SDS-PAGE. Concentration of TK was determined spectrophotometrically at 280 nm using $A^{1\%}_{1cm} = 14.5$ [12]. To obtain holoTK, the enzyme solution was supplemented with 2.5 mM $CaCl_2$, 60 μM ThDP, or 200-250 μM 4′-methylamino-ThDP.

Determination of ThDP and its analog. The concentration of ThDP and 4′-methylamino-ThDP was determined spectrophotometrically using molar absorption coefficients $\varepsilon = 7500\ M^{-1}\cdot cm^{-1}$ at 273.5 nm for ThDP and $\varepsilon = 11{,}900\ M^{-1}\cdot cm^{-1}$ at 265 nm for 4′-methylamino-ThDP [13, 14].

Assaying transketolase activity. Transketolase activity was determined by two methods: (i) by measuring the rate of NAD^+ reduction using GAPD as the coupling enzyme [3] and xylulose-5-phosphate and ribose-5-phosphate as the substrates. The reaction mixture (1 ml) contained 50 mM glycylglycine-HCl, pH 7.6, 10 mM sodium arsenate, 0.37 mM NAD^+, 3 U of GAPD, 3.2 mM dithiothreitol, 2.5 mM $CaCl_2$, 0.2 mM ThDP, 0.25 μg TK, 0.5 mM D-xylulose-5-phosphate, and 1.5 mM D-ribose-5-phosphate. The reaction was initiated by the addition of the substrates. The measurements were made in 1-cm cuvettes at 340 nm using a DW 2000 Aminco spectrophotometer (USA); (ii) by the rate of oxidation of the α-carbanion intermediate in the presence of ferricyanide. The intermediate DHEThDP is oxidized by ferricyanide yielding glycolic acid [4] according to the equation:

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

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

The measurements were made in 1-cm cuvettes using a Uvicon-941 spectrophotometer (Kontron, Germany) in double-beam mode. The initial rate of the reaction was constant during the first 30 sec. Decrease in absorption at 420 nm due to the reduction of ferricyanide was monitored. The reaction mixture (2 ml) contained 50 mM Mops-NaOH, pH 7.6, 2.5 mM $MgCl_2$, 0.1 mM ThDP, 1.25 mM $K_3[Fe(CN)_6]$, 0.5 mM HP, and 50-60 μg TK. For the apoTK–4′-methylamino-ThDP complex, the mixture contained 0.5 mM 4′-methylamino-ThDP and 20 mM HP. The reaction was initiated by the addition of the enzyme.

Differential absorption spectra of transketolase. The spectrum of apoTK (3.6 μM) was recorded using an

Aminco DW-2000 spectrophotometer in 1-cm cuvette containing 50 mM glycylglycine buffer, pH 7.6, and 2.5 mM $CaCl_2$. Then the same cuvette was supplemented with ThDP to achieve the saturated concentration and the spectrum was recorded repeatedly. To record the spectrum of holoTK in the presence of HP, the solution of holoTK was supplemented with 2.5 mM HP. The spectra of ThDP and HP were recorded separately under the same conditions. The differential spectra were obtained by the subtraction of the apoTK and ThDP spectra from the holoTK spectrum, or by the subtraction of the apoTK, ThDP, and HP spectra from the spectrum of holoTK in the presence of the substrate.

Monitoring of intermediate formation using stopped-flow spectroscopy. It was shown previously that the kinetics of the substrate (HP) transformation by native TK could be monitored by measuring the absorption change at 300 nm [15]. Similarly, HP transformation by TK in the complex with 4′-methylamino-ThDP can be monitored by the absorption change at 320 nm. Measurements were made in a 1-cm quartz cuvette using an SX.18MV spectrophotometer (Applied Photophysics, England) at 25°C. The reaction was started by mixing equal volumes of solutions using two syringes. The first syringe contained the enzyme in 25 mM glycylglycine buffer, pH 7.6, 2.5 mM $CaCl_2$, and 50 μM ThDP (or 250 μM 4′-methylamino-ThDP), and the second syringe contained different concentrations of the substrate (HP) in the same buffer. The final concentration of TK active sites was 13.4 μM for native holoTK and 37 μM for the complex of TK with 4′-methylamino-ThDP.

CD spectra. CD spectra were recorded in 1-cm cuvettes at 20°C using a Jasco J810 or Mark V (Jobin Ivon, France) dichrograph interfaced with a computer. The medium contained 50 mM glycylglycine buffer, pH 7.6, 2.5 mM $CaCl_2$, 5.5 μM TK (active site concentration), and 40 μM ThDP or 250 μM 4′-methylamino-ThDP. Substrate concentrations constituted 3.5 mM for HP and 10 mM for GA. After the addition of the substrates, the samples were incubated for 5 min.

Identification of product of one-substrate TK reaction with 4′-methylamino-ThDP as coenzyme by NMR spectroscopy. ApoTK (20.3 μM active sites) was incubated in 50 mM glycylglycine buffer, pH 7.6, containing Ca^{2+}, 120 μM 4′-methylamino-ThDP, and 20 mM HP for 60 min. To separate the protein, the solution was concentrated using a 0.5-ml Vivaspin (Sartorius, Germany) centrifugal filter. The sample was analyzed by ^{1}H-NMR spectroscopy at 300 K using a Bruker™ Avance 400 NMR spectrometer at 400.13 MHz. Before the experiment, the sample was supplemented with 10% (v/v) D_2O (99.9%). Data were analyzed as described previously [16].

RESULTS AND DISCUSSION

Kinetic parameters of transketolase with ThDP and 4′-methylamino-ThDP. Catalytic activity of TK with the native coenzyme and its analog was determined by two methods: by the rate of the formation of glyceraldehyde-3-phosphate in the system with the coupling enzyme and in the oxidative ferricyanide reaction using HP as the donor substrate without acceptor substrate.

As seen from the data presented in table, methylation of the amino group of the coenzyme significantly decreases its affinity to apoTK: dissociation constants for ThDP and its analog in the presence of Ca^{2+} differ more than 10-fold. The specific activity in the standard system containing xylulose-5-phosphate and ribose-5-phosphate as the substrates and GAPD as the coupling enzyme decreases 70-fold in the presence of the ThDP analog. However, the enzyme activity in the complex with 4′-methylamino-ThDP in the one-substrate reaction with ferricyanide (under saturating concentration of HP) 8-fold exceeds the activity with ThDP. It should be noted that the affinity of the TK–ThDP and TK–4′-methylamino-ThDP complexes to the substrate (HP) significantly differs. In the presence of 4′-methylamino-ThDP, the K_m value for HP was found to be 7 mM, this exceeding 100-fold the analogous parameter determined for the native holoTK (70 μM).

CD spectra of transketolase reconstituted with ThDP and 4′-methylamino-ThDP. Binding of ThDP to apoTK results in the appearance of a new band in the CD spectrum of the enzyme with maximum at 320 nm that is absent in the original spectra of the original components.

Kinetic parameters of the transketolase reaction with ThDP and 4′-methylamino-ThDP as the coenzymes (in presence of 2.5 mM Ca^{2+})

Coenzyme	K_d, μM	Activity*, U/mg	Activity**, U/mg	K_m for HP**, μM
ThDP	0.03 [17] 0.25 [17]	37	0.75	70
4′-Methylamino-ThDP	14 [1, 2]	0.52	6	7000

* The transketolase activity determined in the two-substrate reaction.
** The activity and K_m value for HP determined in the one-substrate reaction with ferricyanide.

This band (spectrum *2* in Fig. 2) characterizes the formation of the catalytically active holoenzyme. The addition of HP (donor substrate) results in the inversion of the band (spectrum *3* in Fig. 2) due to the cleavage of the substrate and formation of the intermediate of the transketolase reaction, DHEThDP. The subsequent addition of the acceptor substrate results in the virtually complete restoration of the band intensity (spectrum *4* in Fig. 2). Thus, CD spectra allow separation of different steps of the transketolase reaction: the binding and cleavage of the donor substrate yielding the intermediate DHEThDP and the transfer of the two-carbon fragment to the acceptor substrate.

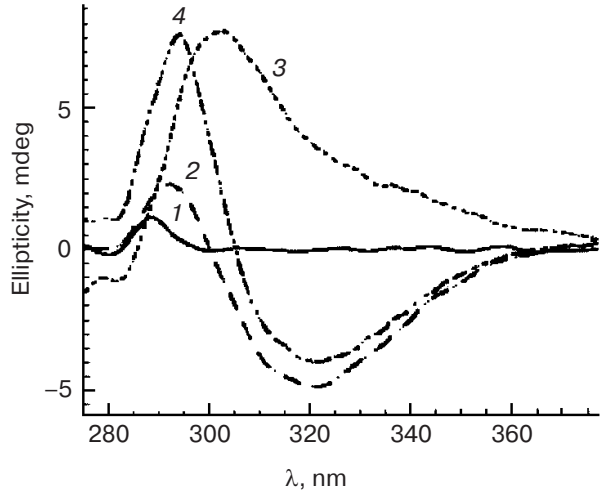


Fig. 2. CD spectra of apoTK (*1*), holoTK (*2*), holoTK + HP (*3*), and holoTK + HP + GA (*4*).

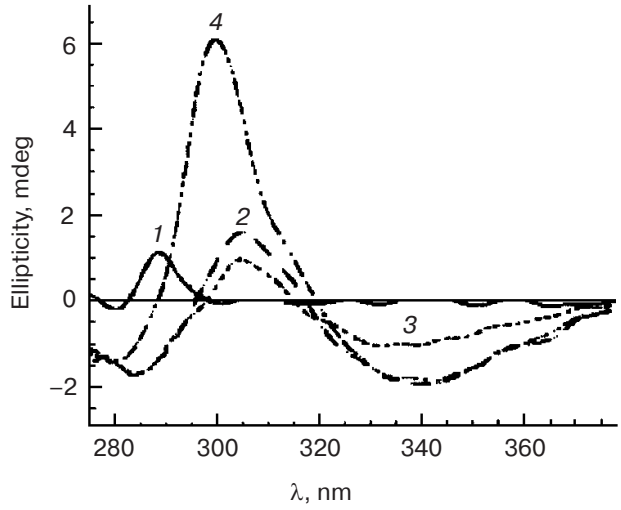


Fig. 3. CD spectra of apoTK (*1*), apoTK–4′-methylamino-ThDP (*2*), apoTK–4′-methylamino-ThDP + HP (*3*), and apoTK–4′-methylamino-ThDP + HP + GA (*4*).

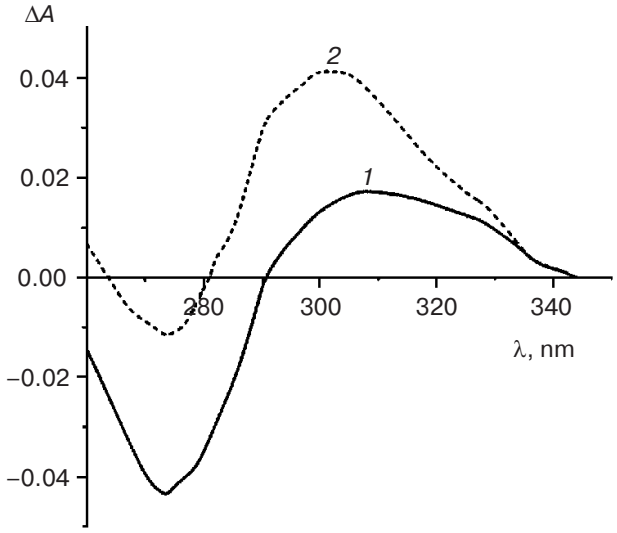


Fig. 4. Differential absorption spectra of holoTK in the absence (*1*) and in the presence (*2*) of 2.5 mM hydroxypyruvate: 50 mM glycylglycine, pH 7.6, 2.5 mM $CaCl_2$, 0.13 mM ThDP, 3.61 μM TK.

The reconstitution of TK with the methylated coenzyme analog 4′-methylamino-ThDP shifts the maximum of the band corresponding to the holoenzyme by 20 nm to longer wavelength, decreasing its amplitude compared to the native holoenzyme (spectrum *2* in Fig. 3). Nevertheless, the changes in the spectrum corresponding to both steps of the transketolase reaction that are characteristic for the native holoenzyme (Fig. 2) were also detected with the methylated analog: cleavage of the donor substrate with the formation of the intermediate (spectrum *3* in Fig. 3) and the transfer of the two-carbon fragment onto the acceptor substrate (spectrum *4* in Fig. 3).

Registration of formation and breakdown of intermediate of the transketolase reaction using stopped-flow spectroscopy. The changes observed in the CD spectra are accompanied by corresponding changes in the absorption spectra of the protein [15]. Figure 4 presents the differential absorption spectrum of holoTK in the absence (curve *1*) and in the presence of HP (curve *2*). The broad absorption band in the presence of HP is due to the formation of DHEThDP. This fact allows registration of the binding and cleavage of the donor substrate yielding the intermediate DHEThDP (or DHE-4′-methylamino-ThDP) by the changes in the absorption spectrum.

The rate of the formation and stability of DHEThDP and DHE-4′-methylamino-ThDP were investigated by stopped-flow spectroscopy assay by measuring the absorption change at 300 nm in the case of ThDP and at 320 nm in the case of 4′-methylamino-ThDP. The wavelengths were chosen experimentally as corresponding to the maximal absorption changes during the formation of the intermediate of the transketolase reaction in the first and the second cases, respectively.

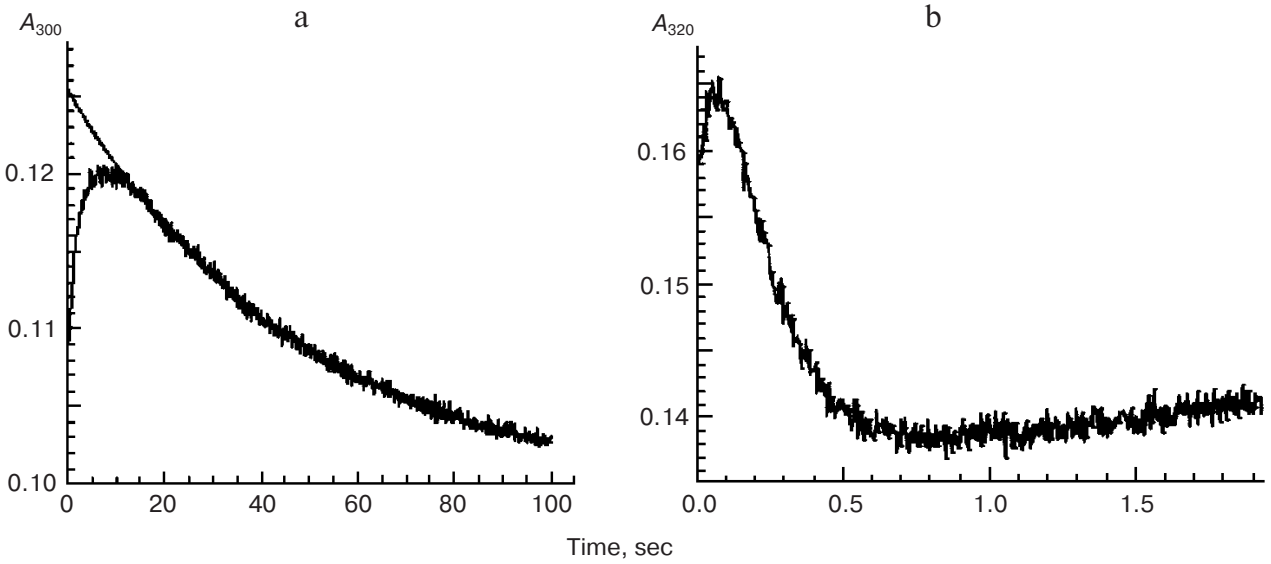


Fig. 5. Monitoring of HP transformation during one-substrate reaction using stopped-flow spectroscopy. a) Native holoTK: the reaction mixture contained equal concentrations (13.4 μM) of the enzyme active sites and HP (single-turnover conditions), 25 mM glycylglycine buffer, pH 7.6, 2 mM $CaCl_2$, and 50 μM ThDP. b) Complex apoTK–4′-methylamino-ThDP: the reaction mixture contained 37 μM enzyme active sites, 5 mM HP, 25 mM glycylglycine buffer, pH 7.6, 2 mM $CaCl_2$, and 200 μM 4′-methylamino-ThDP. The temperature was 25°C, optical path length 1 cm.

Fast kinetics of the intermediate formation after the addition of HP to the complexes of apoTK with ThDP and apoTK with ThDP analog is presented in Figs. 5a and 5b, respectively. The formation of the intermediate in the case of the native holoenzyme was investigated in the presence of equimolar concentrations of TK active sites and the substrate (Fig. 5a) and under saturating concentration (4 mM) of the substrate (data not shown). It is seen that the absorption curve exhibits two-phase character: first the absorption grows (first phase), reaching the maximal value in 8 sec, and then decreases (second phase). The apparent rate constant for the first phase depends on HP concentration and reaches its maximal value (45 sec^{-1}) at 4 mM HP (data not shown). The rate constant for the second phase does not depend on the substrate concentration and constitutes 0.02 sec^{-1}.

Transformation of HP in the presence of the apoTK–4′-methylamino-ThDP complex (Fig. 5b) was investigated by measuring the absorption changes at 320 nm. In these experiments, HP concentration varied from 2 to 20 mM. Figure 5b presents the data of the experiment where HP concentration was 5 mM. As in the case of the native holoenzyme, the curve has two-phase character. After the initial increase in the absorption (first phase), the absorption decreases (second phase). The subsequent repeated increase in the absorption is due to the formation of the reaction product (see below). The rate constant of the first phase depends on the substrate concentration and reaches maximal value (45 sec^{-1}) at saturating HP concentration (data not shown). The rate constant of the second phase does not depend on the substrate concentration and constitutes 6 sec^{-1}. Comparison of the two-phase kinetics of the substrate transformation by the native holoenzyme and by the complex of apoTK with ThDP analog indicates that the rate constants of the first phase are equal, but the rate constants of the second phase significantly differ.

Identification of product of hydroxypyruvate transformation by holotransketolase. The ^{1}H-NMR spectroscopy assay detected only one intermediate of the equimolar HP transformation by native holotransketolase, DHEThDP (Fig. 6). Thus, the first phase of the reaction that is accompanied by an increase in the absorption at 300 nm (Fig. 5a) reflects the formation of the covalent enzyme–substrate complex with its subsequent decarboxylation yielding the key intermediate of the transketolase reaction, DHEThDP. The fact that the same amount of DHEThDP was revealed in the second phase suggests that the second (slow) phase of the reaction reflects the protonation of DHEThDP, but not its breakdown with the yield of free ThDP and glycolaldehyde. The ^{1}H-NMR spectroscopic analysis does not discriminate α-carbanion/enamine from the protonated form, since after the acidification of the medium (to stop the reaction) only the protonated form of DHEThDP can be revealed. The data of time-resolved X-ray study are in agreement with the presented results (Fig. 5a), detecting DHEThDP in the active site of holoTK in the presence of HP [6].

The presented data indicate that the rate constants of the intermediate formation by native holoTK and by the complex of the apoenzyme with 4′-methylamino-ThDP are identical (45 sec^{-1}), but the rate of the intermediate protonation is significantly faster in the complex with 4′-methylamino-ThDP (6 sec^{-1} compared with 0.02 sec^{-1}). Thus, it can be assumed that the different rate of proto-

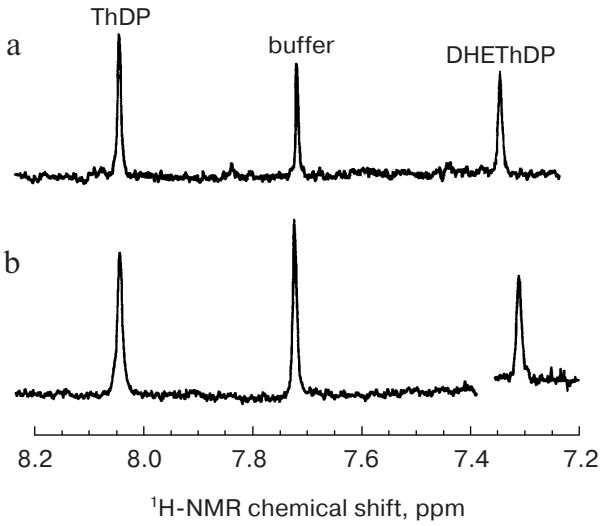


Fig. 6. Identification of intermediate of transketolase one-substrate reaction with HP as substrate under single-turnover conditions. To obtain holoenzyme, apoTK (219 μM active sites) was incubated with 3 mM Mg^{2+} and 219 μM ThDP in 20 mM potassium-phosphate buffer, pH 7.6, for 5 min. Then the donor substrate (HP) was added to final concentration of 110 μM. According to the data presented in Fig. 5a, after 8 (a) or 40 sec (b), the reaction was stopped by the addition of trichloroacetic acid. The samples were prepared and analyzed as indicated in "Materials and Methods" according to [16].

nation is due to the different resonance stabilization of the α-carbanion/enamine intermediate. It is known that the 4′-NH_2-group of ThDP is involved in stabilization of the intermediate of the transketolase reaction, forming a hydrogen bond with the oxygen of the α-hydroxyl-group of DHEThDP (Fig. 1) [7]. The methylation of the 4′-amino group of ThDP is likely to prevent this stabilization, increasing the rate of protonation and oxidation in the presence of ferricyanide.

Investigation of intermediate stability by CD spectroscopy. To test the assumption that the ThDP amino group is involved in stabilization of the enamine form of the intermediate and therefore any modification (for example, methylation) causes the opposite effect, we investigated the stability of the intermediate DHE-4′-methylamino-ThDP by CD spectroscopy. As mentioned above, CD spectra of the TK complexes allow monitoring of two stages of the two-substrate transketolase reaction both with the native holoenzyme and with the holoenzyme containing in the active site the methylated ThDP analog, 4′-methylamino-ThDP (Figs. 2 and 3). A significant difference in the spectra is observed in the case of the transketolase one-substrate reaction, when the reaction proceeds in the presence of the donor substrate without acceptor substrate. A prolonged incubation of the TK–4′-methylamino-ThDP complex with the donor substrate leads to the appearance of a negative band with maximum at 310-312 nm, whose amplitude grows with time (spectra *3-8* in Fig. 7). The removal of the protein does not affect the intensity of the band. This indicates that the band is due to the formation of the chiral product of the one-substrate transketolase reaction that is released into the medium, but not to the intermediate bound to the protein. In control experiments in the absence of TK no changes were observed in the indicated region of the CD spectrum, indicating that the chiral product that was identified by the characteristic changes in the CD spectrum was formed enzymatically.

It is noteworthy that in the experiments with the holoenzyme containing the native coenzyme, reaction product with maximum at 310-312 nm is not formed.

Analysis of product of HP transformation by TK–4′-methylamino-ThDP complex. ApoTK was incubated in 50 mM glycylglycine buffer, pH 7.6, containing 2.5 mM Ca^{2+}, 120 μM 4′-methylamino-ThDP, and 20 mM HP for 60 min. After the removal of the protein, the reaction mixture was analyzed by ^{1}H-NMR spectroscopy. It was shown that approximately 60% of the added methylated analog of ThDP was transformed into DHE-4′-methylamino-ThDP (Fig. 8). No other possible products of the one-substrate transketolase reaction were detected in the reaction mixture. The presented data indicate that TK in the complex with 4′-methylamino-ThDP possesses DHE-4′-methylamino-ThDP-synthase activity. The rate of this reaction (monitored by absorption change at 310 nm) depends on both TK concentration (in the range of 0.5-1.75 mg/ml) and the substrate (HP) concentration (6-36 mM) (data not shown).

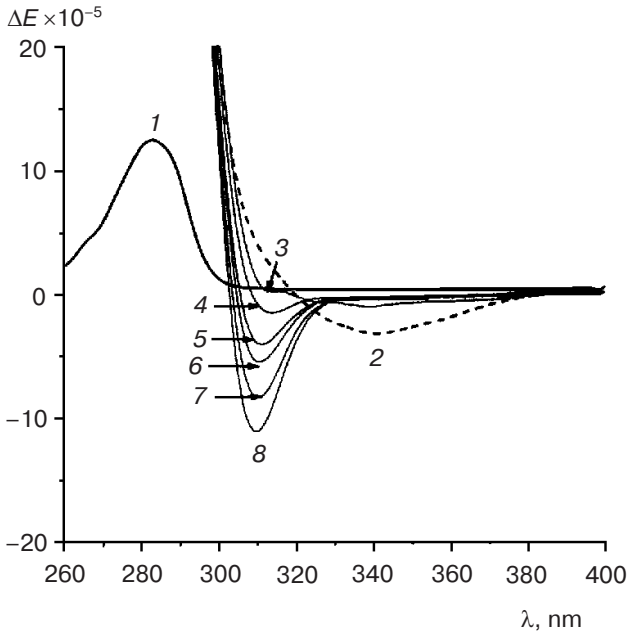


Fig. 7. CD spectra of apoTK (*1*), apoTK–4′-methylamino-ThDP (*2*), and apoTK–4′-methylamino-ThDP + HP (*3*); *4-8*) same as (*3*) after 5, 10, 15, 20, and 25 min of incubation, respectively.

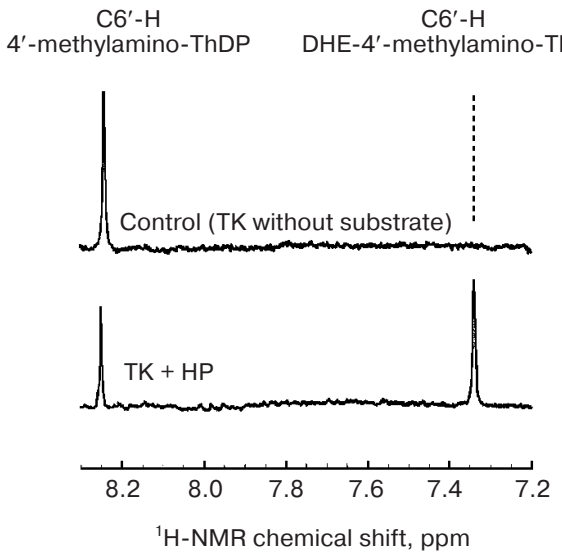


Fig. 8. Analysis of reaction products by ^{1}H-NMR spectroscopy. ApoTK was incubated in 50 mM glycylglycine buffer, pH 7.6, in the presence of 2.5 mM Ca^{2+}, 120 μM 4′-methylamino-ThDP, and 20 mM HP for 60 min. After the removal of the protein, the reaction mixture was analyzed by ^{1}H-NMR spectroscopy.

The experimental data presented above demonstrate that modification of the amino group of ThDP (in this case, methylation) destabilizes the central intermediate of the transketolase reaction, α-carbanion/enamine of DHEThDP. Due to this fact, the use of 4′-methylamino-ThDP as the coenzyme significantly increases the rate of the one-substrate transketolase reaction in the presence of ferricyanide, where the α-carbanion intermediate is subjected to oxidation (table). Besides, the rate of protonation of the formed intermediate increases (see Figs. 5a and 5b). As a result, optically active DHE-4′-methylamino-ThDP is formed that can be detected by CD spectroscopy (Fig. 7) and ^{1}H-NMR analysis (Fig. 8). The protonated form of DHE-4′-methylamino-ThDP is released from the active sites of TK and accumulated in the medium on preparative scale. Thus, we revealed DHE-4′-methylamino-ThDP-synthase activity of TK that is not observed while using native ThDP as the coenzyme.

Therefore, the 4′-amino group of the coenzyme not only promotes deprotonation of the second carbon atom of the thiazole ring [13, 14, 18-20], this providing the thiamine catalysis, but also stabilizes the intermediate of the transketolase reaction, dihydroxyethyl-thiamine diphosphate.

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

REFERENCES

1. Pustynnikov, M. G., Neef, H., Usmanov, R. A., Schellenberger, A., and Kochetov, G. A. (1986) *Biokhimiya*, **51**, 1003-1016.
2. Usmanov, R. A., Neef, H., Pustynnikov, M. G., Schellenberger, A., and Kochetov, G. A. (1985) *Biochem. Int.*, **10**, 479-486.
3. Kochetov, G. A. (1982) *Meth. Enzymol.*, **90**, 209-223.
4. Usmanov, R. A., and Kochetov, G. A. (1981) *Biochem Int.*, **3**, 33-39.
5. Solovjeva, O. N., Bykova, I. A., Meshalkina, L. E., Kovina, M. V., and Kochetov, G. A. (2001) *Biochemistry (Moscow)*, **66**, 932-936.
6. Bykova, I. A., Solovjeva, O. N., Meshalkina, L. E., Kovina, M. V., and Kochetov, G. A. (2001) *Biochem. Biophys. Res. Commun.*, **280**, 845-847.
7. Fiedler, E., Thorell, S., Sandalova, T., Golbik, R., Konig, S., and Schneider, G. (2002) *Proc. Natl. Acad. Sci. USA*, **99**, 591-595.
8. Schellenberger, A., and Winter, K. (1966) *Hoppe-Seylers Z. Phys. Chem.*, **344**, 16-24.
9. Schellenberger, A., Muller, V., Winter, K., and Hubner, G. (1966) *Hoppe-Seylers Z. Phys. Chem.*, **344**, 244-260.
10. Wikner, C., Meshalkina, L., Nilsson, U., Nikkola, M., Lindqvist, Y., Sundstrom, M., and Schneider, G. (1994) *J. Biol. Chem.*, **269**, 32144-32150.
11. Golbik, R., Meshalkina, L. E., Sandalova, T., Tittmann, K., Fiedler, E., Neef, H., Konig, S., Kluger, R., Kochetov, G. A., Schneider, G., and Hubner, G. (2005) *FEBS J.*, **272**, 1326-1342.
12. Heinrich, C. P., Noack, K., and Wiss, O. (1972) *Biochem. Biophys. Res. Commun.*, **49**, 1427-1432.
13. Schellenberger, A., and Hubner, G. (1965) *Hoppe Seylers Z. Physiol. Chem.*, **343**, 189-192.
14. Schellenberger, A., Hubner, G., and Neef, H. (1997) *Meth. Enzymol.*, **279**, 131-146.
15. Kochetov, G. A., Usmanov, R. A., and Mevkh, A. T. (1973) *Biochem. Biophys. Res. Commun.*, **54**, 1619-1626.
16. Tittmann, K., Golbik, R., Uhlemann, K., Khailova, L., Schneider, G., Patel, M., Jordan, F., Chipman, D. M., Duggleby, R. G., and Hubner, G. (2003) *Biochemistry*, **42**, 7885-7891.
17. Kochetov, G. A., Tikhomirova, N. K., and Philippov, P. P. (1975) *Biochem. Biophys. Res. Commun.*, **63**, 924-930.
18. Schellenberger, A. (1967) *Angewandte Chemie* (International Edition), **6**, 1024-1035.
19. Schellenberger, A. (1982) *Ann. N. Y. Acad. Sci.*, **378**, 51-62.
20. Schellenberger, A. (1998) *Biochim. Biophys. Acta – Protein Struct. Mol. Enzymol.*, **1385**, 177-186.