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Influence of Donor Substrate on Kinetic Parameters of Thiamine Diphosphate Binding to Transketolase

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Abstract—The two-step mechanism of interaction of thiamine diphosphate (ThDP) with transketolase (TK) has been studied: TK + ThDP ↔ TK···ThDP ↔ TK*–ThDP. The scheme involves the formation of inactive intermediate complex TK···ThDP followed by its transformation into catalytically active holoenzyme, TK*–ThDP. The dissociation and kinetic constants for individual stages of this process have been determined. The values of forward and backward rate constants change in the presence of the donor substrate hydroxypyruvate. This finally leads to an increase in the overall affinity of the coenzyme to TK.

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Transketolase (TK, EC 2.2.1.1) is a key enzyme of the non-oxidative branch of the pentose phosphate pathway of carbohydrate conversion, which, together with transaldolase, provides a link between the pentose phosphate pathway and glycolysis [1]. TK catalyzes a reversible transfer of a two-carbon fragment (glycol aldehyde residue) from ketose (donor substrate) to aldose (acceptor substrate). The transketolase reaction is irreversible only when hydroxypyruvate (HP) is used as a donor substrate, since its reaction results in the formation of CO_2 [2].

Transketolase is characterized by broad specificity. The function of donor substrate can be performed by xylulose 5-phosphate, sedoheptulose 7-phosphate, fructose 6-phosphate, HP, and other compounds; the function of acceptor substrate can be performed by ribose 5-phosphate, glycol aldehyde, glyceraldehyde 3-phosphate, and so on. The cofactors of TK are thiamine diphosphate (ThDP) and bivalent metal ions, such as Ca^{2+} and Mg^{2+} [3].

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

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Transketolase from *Saccharomyces cerevisiae* is the simplest representative of ThDP-dependent enzymes. According to X-ray data, the transketolase molecule consists of two identical subunits having a molecular weight of 74 kD [4]. The enzyme molecule contains two active sites characterized by identical catalytic activity [5, 6]. In native holoenzyme, one calcium ion is found in each active site [7], but until recently magnesium ions were mainly used as cofactors for determination of enzyme activity. Indeed, such a replacement has no effect on measured activity, but, as we will see below, it is significant for the interaction of ThDP with the apoprotein.

The coenzyme in holoTK is located in a cleft between the two subunits [8]. Both subunits are involved in its binding to the protein. The pyrophosphate group of the coenzyme interacts with the protein both directly (through hydrogen bonds with histidine 69 and 263 as well as with glycine 158) and indirectly through the calcium ion. The thiazole ring of the coenzyme is bound with the two subunits mainly due to hydrophobic interactions [9]. The aminopyrimidine ring in the holoenzyme is located in a hydrophobic pocket formed by aromatic amino acid residues [10].

$$TK + ThDP \overset{K_d}{\rightleftharpoons} TK\cdots ThDP \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} TK^*{-}ThDP$$

Two-stage mechanism of ThDP interaction with apoTK (shown for a single active site)

Scheme 1

ThDP binding with active sites of TK involves at least two stages (Scheme 1) [11, 12]. First, catalytically inactive intermediate complex TK···ThDP is formed. This stage is fast and reversible. The second stage is slow and accompanied by conformational changes in the protein molecule, resulting in the formation of fairly stable, catalytically active holoenzyme TK*–ThDP.

It is noteworthy that the formation of catalytically active holoenzyme resulting from binding of coenzyme to apoprotein is accompanied by the appearance of an induced band in the TK absorption spectrum with a maximum at 320 nm, which is absent in the initial components [13, 14]. The intensity of the band strictly correlates with the amount of formed catalytically active holoenzyme [5, 15]. This provided the opportunity to study the interaction between coenzyme and apoprotein and the effect of different ligands and cofactors using spectrophotometric titration of apoTK with thiamine diphosphate [16-18].

The active TK sites are not equivalent in terms of ThDP binding if Ca^{2+} is used as a cofactor in experiments. Negative cooperativity is observed [19-22]. However, in the presence of Mg^{2+} (instead of Ca^{2+}), negative cooperativity is not found at all [23, 24] or is slight [22].

In the presence of a donor substrate, the affinity of ThDP to apoTK is increased irrespectively of which cation is used as the cofactor, Ca^{2+} or Mg^{2+} [25]. An assumption has been made [26] that the effect of substrate is directed to a stage of reverse conformational transition characterized by constant k_{-1} (see Scheme 1).

The aim of this work was to determine the precise values of equilibrium and kinetic constants for individual stages of the interaction between ThDP and apoTK and to find out what stages and to what extent they are influenced by a donor substrate of transketolase reaction. For this reason we have developed a theoretical model for the interaction between ligand-binding sites in the dimeric protein molecule [27].

THEORETICAL ANALYSIS OF INTERACTION BETWEEN THIAMINE DIPHOSPHATE-BINDING SITES OF TRANSKETOLASE

Earlier the kinetic model for binding of ThDP to transketolase taking into account the two-stage mechanism (Scheme 1) and cooperative interaction of two identical active sites has been suggested (Scheme 2).

The two ThDP-binding sites in the TK molecule are structurally equivalent. Therefore, during the first stage (characterized by dissociation constant K_d) ThDP binds with equal probability to either of them. As a result, TEE or EET complexes are formed, which, in principle do not differ from each other. The question is only, which of the two active centers binds ThDP, nominally the first or the second. The same relates to the enzyme forms $E\bar{E}T$ and $T\bar{E}E$ generated as a result of conformational changes in the active sites caused by primary binding of ThDP (Scheme 2). Equilibrium isomerization constant K_1 is not dependent on whether the neighboring ThDP-binding site of the other active center is occupied or not. The state of the enzyme active centers E in the forms $T\bar{E}E$ and $E\bar{E}T$ is characterized by the same affinity (K_d) to ThDP as the active sites in the initial form EE. However, it is assumed that the presence of conformationally altered active center in a dimer influences the conformational state of the other center (in other words, isomerization constant K_2 is different from isomerization constant K_1).

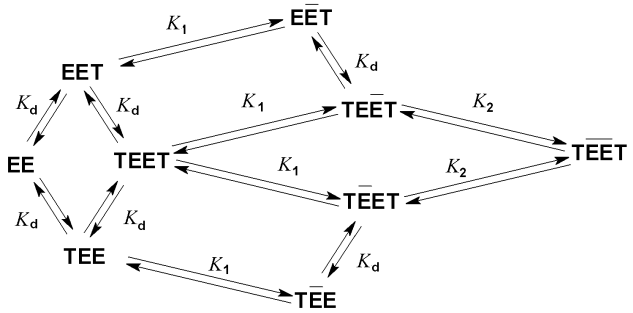
For the discussed scheme the equilibrium constants will be described by the following equations:

$$K_d = \frac{[EE][T]}{[EET]} = \frac{[EET][T]}{[TEET]} = \frac{[E\bar{E}T][T]}{[T\bar{E}\bar{E}T]}, \quad (1)$$

$$K_1 = \frac{[EET]}{[E\bar{E}T]} = \frac{[TEET]}{[T\bar{E}\bar{E}T]}, \quad (2)$$

$$K_2 = \frac{[T\bar{E}\bar{E}T]}{[T\bar{E}\bar{E}T]}, \quad (3)$$

where [T] is the concentration of free ThDP.



Interaction of ThDP with apotransketolase. E, apoTK active site; EE, initial catalytically inactive apoenzyme dimer; T, thiamine diphosphate; $E\bar{E}$ and $\bar{E}E$, dimeric enzyme forms containing one conformationally altered active site ($\bar{E}$); $\bar{E}\bar{E}$, dimeric enzyme form with two conformationally altered active sites [28]

Scheme 2

Concentrations of ThDP-containing forms are expressed through the concentration of the initial enzyme form [EE] by the following equations:

$$[EET] = \frac{[EE][T]}{K_d}, \quad (4)$$

$$[TEET] = \frac{[EET][T]}{K_d} = \frac{[EE][T]^2}{K_d^2}, \quad (5)$$

$$[E\bar{E}T] = \frac{[EET]}{K_1} = \frac{[EE][T]}{K_1 K_d}, \quad (6)$$

$$[T\bar{E}ET] = \frac{[E\bar{E}T][T]}{K_d} = \frac{[EE][T]^2}{K_1 K_d^2}, \quad (7)$$

$$[T\bar{\bar{E}}ET] = \frac{[TE\bar{E}T]}{K_2} = \frac{[EE][T]^2}{K_2 K_1 K_d^2}. \quad (8)$$

The character of the interaction between ThDP-binding sites is determined by the relationship between the constants K_1 and K_2. The condition $K_2 > K_1$ corresponds to negative cooperative interactions between ThDP-binding sites. The condition $K_2 < K_1$ accounts for positive cooperative interactions.

The degree of saturation (Y) of dimeric TK with thiamine diphosphate is determined as the ratio of the sum of ThDP-containing enzyme forms to the total dimer concentration:

$$Y = \frac{[EET] + [TEE] + [E\bar{E}T] + [T\bar{E}E] + [T\bar{E}ET] + [TE\bar{E}T] + [T\bar{\bar{E}}ET]}{2[E]_0} =$$

$$= \left[\left(1 + \frac{1}{K_1}\right)\frac{[T]}{K_d} + \left(1 + \frac{2}{K_1} + \frac{1}{K_1 K_2}\right)\frac{[T]^2}{K_1 K_d^2}\right]\left[1 + \right.$$

$$\left. + \left(1 + \frac{1}{K_1}\right)\frac{2[T]}{K_d} + \left(1 + \frac{2}{K_1} + \frac{1}{K_1 K_2}\right)\frac{[T]^2}{K_d^2}\right]^{-1}, \quad (9)$$

where $[E]_0$ is the total enzyme concentration calculated per active center. The Y value varies between 0 and 1 with increase in the concentration of free ThDP (the limiting value of Y at $[T] \to \infty$ is equal to 1).

The degree of conformational changes (Z) in the enzyme is calculated as the ratio of the sum of concentrations of conformationally altered active sites to the total enzyme concentration:

$$Z = \frac{[E\bar{E}T] + [T\bar{E}E] + [T\bar{E}ET] + [TE\bar{E}T] + [T\bar{\bar{E}}ET]}{2[E]_0} =$$

$$= \left[\frac{[T]}{K_1 K_d} + \left(1 + \frac{1}{K_2}\right)\frac{[T]^2}{K_1 K_d^2}\right]\left[1 + \left(1 + \frac{1}{K_1}\right)\frac{2[T]}{K_d} + \right.$$

$$\left. + \left(1 + \frac{2}{K_1} + \frac{1}{K_1 K_2}\right)\frac{[T]^2}{K_d^2}\right]^{-1}. \quad (10)$$

Upon $[T] \to \infty$, the Z value approaches the limit:

$$Z_{lim} = \frac{1 + K_2}{1 + 2K_2 + K_1 K_2}. \quad (11)$$

Normalized Z value will look like:

$$Z_{norm} = \frac{Z}{Z_{lim}} =$$

$$= \left\{\left(1 + 2K_2 + K_1 K_2\right)\left[\frac{[T]}{K_1 K_d} + \left(1 + \frac{1}{K_2}\right)\frac{[T]^2}{K_1 K_d^2}\right]\right\}\left\{\left(1 + K_2\right)\left[1 + \right.\right.$$

$$\left.\left. + \left(1 + \frac{1}{K_1}\right)\frac{2[T]}{K_d} + \left(1 + \frac{2}{K_1} + \frac{1}{K_1 K_2}\right)\frac{[T]^2}{K_d^2}\right]\right\}^{-1}. \quad (12)$$

The value of Z_{norm} varies between 0 and 1 with an increase in ThDP concentration.

By way of example, let us consider the case of negative cooperativity ($K_2 > K_1$). Figures 1a and 1b show the dependence of the degree of saturation (Y) and conformational changes (Z) on the concentration of free ThDP [T] calculated for the following parameter values: K_d = 1 mM; $K_1 = 0.01$; $K_2 = 0.1$. For the curve shown in Fig. 1a the [T] value that corresponds to $Y = 0.5$ ($[T] = [T]_{0.5}$) was found to be equal to 30.1 μM. To demonstrate the deviation of a theoretical curve from a hyperbola, we plotted a curve (dashed line in Fig. 1a) described by an equation of hyperbolic dependence:

$$Y_{hyp} = \frac{[T]/[T]_{0.5}}{1 + [T]/[T]_{0.5}}. \quad (13)$$

As can be seen from Fig. 1a, a theoretical curve corresponding to negative cooperativity is characterized by a lower slope of the inflection point compared to a hyperbolic curve (dashed line). As seen from Fig. 1b, the limit of Z reached at $[T] \to \infty$ has a value less than 1: Z_{lim} =

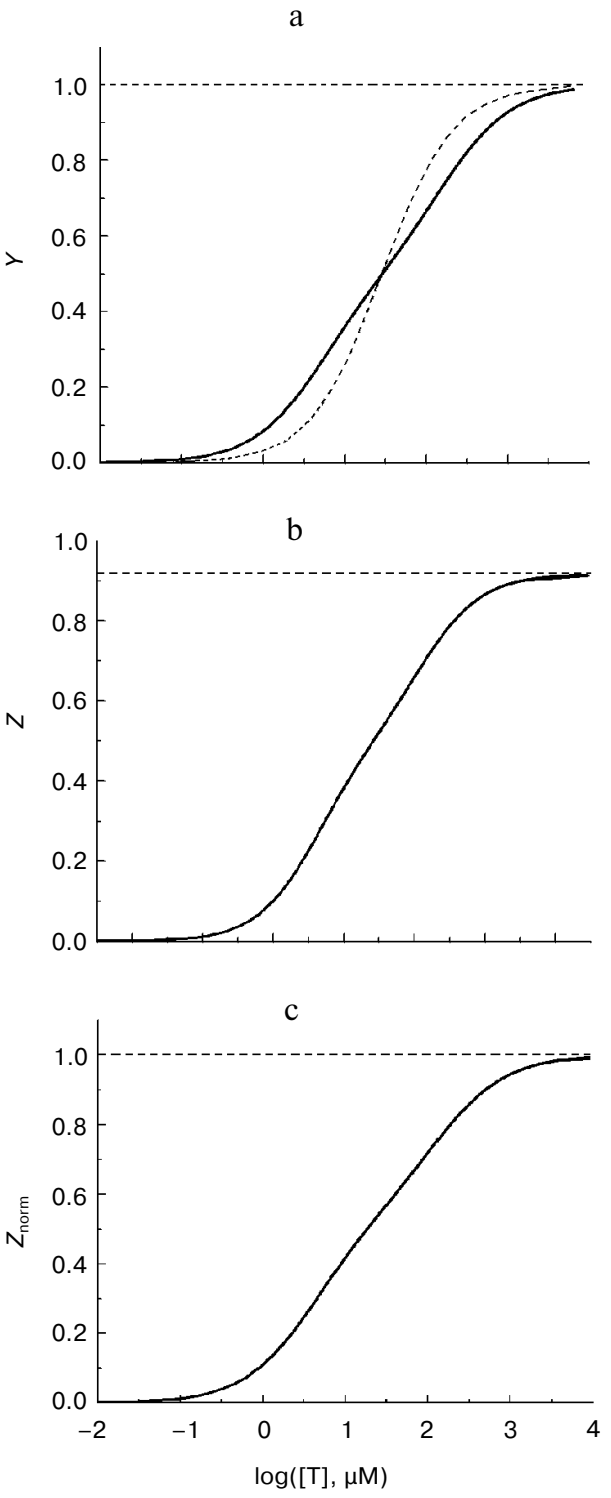


Fig. 1. Theoretical dependence of the degree of saturation of apoTK with thiamine diphosphate (Y) (a), degree of conformational changes in enzyme (Z) (b), and normalized Z value ($Z_{norm} = Z/Z_{lim}$) (c) on the logarithm of free thiamine diphosphate concentration (T) calculated from the Eqs. (9), (10), and (12) using the following parameter values: $K_d = 1$ mM, $K_1 = 0.01$, $K_2 = 0.1$. The dashed line in panel (a) corresponds to a hyperbolic dependence (13) at $[T]_{0.5} = 30.1$ μM.

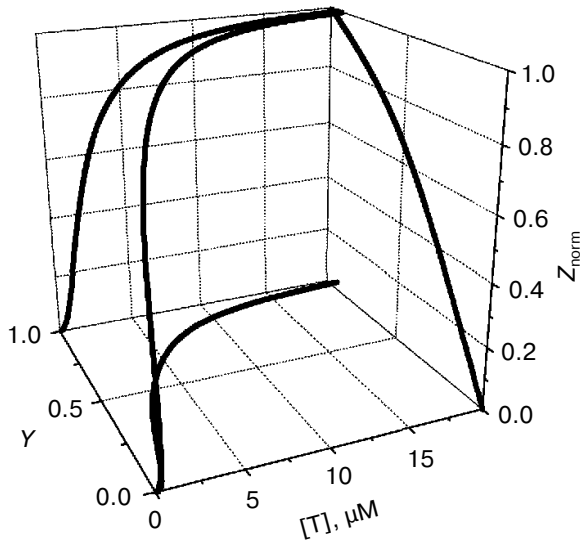


Fig. 2. Three-dimensional graph showing the dependence of saturation degree (Y) and normalized degree of conformational changes (Z_{norm}) on the concentration of free ThDP (T) (K_d = 1 mM, $K_1 = 0.01$, $K_2 = 0.1$).

0.915. Figure 1c shows the dependence of the normalized Z value ($Z_{norm} = Z/Z_{lim}$) on the concentration of free ThDP.

The dependence of Y and Z_{norm} on [T] can be represented as a 3D graph (Fig. 2). The XY projection corresponds to the dependence of Y on [T], projection XZ the dependence of Z_{norm} on [T], and projection YZ the dependence of Z_{norm} on Y.

The construction of the dependence of the degree of conformational changes (Z) on the degree of protein saturation with ligand (Y) provides valuable information for elucidation of a mechanism of cooperative interactions between ligand-binding sites in allosteric enzymes.

DETERMINATION OF EQUILIBRIUM AND KINETIC CONSTANTS FOR INDIVIDUAL STAGES OF THE INTERACTION BETWEEN ThDP AND apoTK USING Mg^{2+} AS A COFACTOR

Figure 3a shows the data on ThDP binding to apoTK in the presence of different enzyme concentrations obtained by the stopped flow method. Figure 3b illustrates the dependence of initial binding rate (v_0, measured as the change in absorbance at 320 nm during the unit of time) on the concentration of ThDP. This dependence is described by a simple hyperbolic function:

$$v_0 = \frac{v_{0,\,\mathrm{lim}}\,[\mathrm{T}]}{K_d + [\mathrm{T}]}\,, \qquad (14)$$

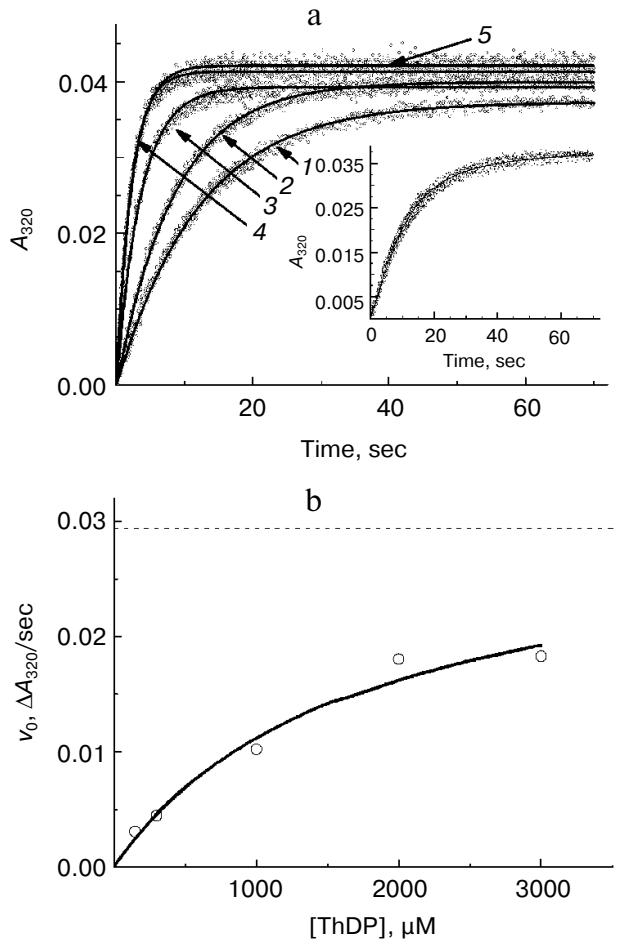


Fig. 3. Kinetics of ThDP binding by transketolase using Mg^{2+} as a cofactor. Conditions: 50 mM glycyl-glycine buffer, pH 7.6, 6.75 μM (dimer) TK, temperature 25°C. a) Kinetic curves were obtained at ThDP concentrations equal to 150 (*1*), 300 (*2*), 1000 (*3*), 2000 (*4*), and 3000 (*5*) μM. The insert shows the kinetics of ThDP binding to transketolase in the presence of 1 mM hydroxypyruvate; [ThDP] = 150 μM [26]. Solid curves were calculated based on Eqs. (18)-(27). b) Dependence of initial rate of the interaction between ThDP and transketolase (v_0) on the initial ThDP concentration. Experimental data are shown as points, and the solid curve was calculated based on Eq. (14). The horizontal dashed line corresponds to the limiting v_0 value at [ThDP] $\to \infty$.

where $v_{0,lim}$ is the limiting value of the initial rate as the ThDP concentration approaches infinity, and K_d is the microscopic dissociation constant for the inactive TK···ThDP complex (Scheme 1). Analysis of the dependence of v_0 on ThDP concentration gives the following parameter values: $v_{0,lim} = 0.0029 \pm 0.0004$ ΔA_{320}/sec and $K_d = 1.6 \pm 0.1$ mM.

The $v_{0,lim}$ characterizes the rate of conformational transition EET $\to$ $\bar{E}$ET (Scheme 3):

$$v_{0,lim} = 2\varepsilon k_{+1}[E]_0, \quad (15)$$

where ε is molar extinction coefficient for the ThDP complex with conformationally altered active site ($\bar{E}$), k_{+1} is the rate constant for conformational transition EET $\to$ E$\bar{E}$T or TEE $\to$ T$\bar{E}$E.

The K_d value calculated based on kinetic data obtained using stopped flow was used by us for the analysis of saturation function of TK with thiamine diphosphate shown in Fig. 4 (curve *1*). To describe the dependence of A_{320} on the concentration of coenzyme, we applied Eqs. (9) and (10). The expression for Z contains the value of free ThDP concentration, [T], which is connected with the total coenzyme concentration, $[T]_{tot}$, by the following equation:

$$[T] = [T]_{tot} - 2\varepsilon[E]_0 Y. \quad (16)$$

The increase in absorbance (A_{320}) during the titration of apoTK with thiamine diphosphate can be calculated from the equation:

$$A_{320} = 2\varepsilon[E]_0 Z. \quad (17)$$

To find the correlation between the theoretical equation and experimental data, we used the computer program Scientist (MicroMath, USA), resulting in the following parameter values: $K_1 = 0.0006 \pm 0.0002$; $K_2 = 0.0066 \pm 0.0007$; $\varepsilon = 0.00269 \pm 0.00002$ $\mu M^{-1} \cdot cm^{-1}$.

Using these values of the above parameters (K_1, K_2, K_d, ε), we performed the analysis of kinetic data on ThDP binding to transketolase shown in Fig. 3a. First, we calculated the value of k_{+1} constant using Eq. (15). It was found to be equal to 1.33 ± 0.2 sec^{-1}. Then, substituting the values of k_{+1} and K_1 in the equation for conformational transition, we determined the rate constant for the reverse conformational transition: $k_{-1} = 0.0008 \pm 0.0001$ sec^{-1}.

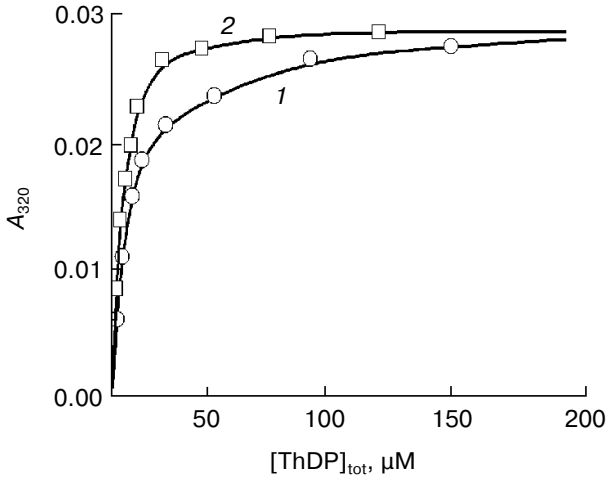
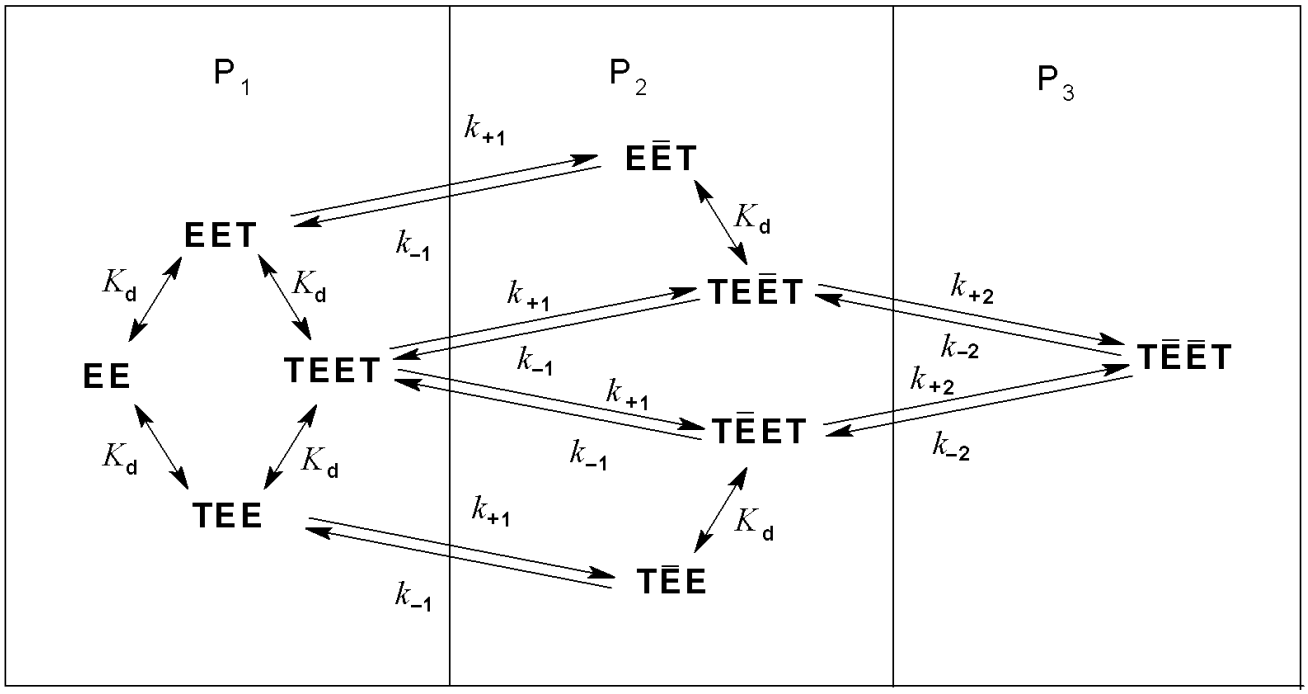


Fig. 4. Titration of apotransketolase with thiamine diphosphate in the absence (*1*) and presence (*2*) of 2.5 mM donor substrate, hydroxypyruvate. Conditions: 50 mM glycyl-glycine buffer, pH 7.6, 2.5 mM $MgCl_2$, apoTK (0.7 mg/ml) [29]. Points show the experimental data, solid curves were calculated from Eqs. (9), (10), (16), and (17).



Kinetic scheme of binding of ThDP to apotransketolase. P_1, P_2, and P_3 are pools of enzyme forms determined by Eqs. (18)-(20)

Scheme 3

To calculate the constants of forward and backward conformational transitions for the second coenzyme-binding active site of the enzyme (k_{+2} and k_{-2}, respectively) we used, according to the Scheme 3, the following kinetic equations. First, we divided all enzyme forms into three pools:

$$P_1 = [EE] + [TEE] + [EET] + [TEE], \quad (18)$$

$$P_2 = [E\bar{E}T] + [T\bar{E}E] + [TE\bar{E}T] + [T\bar{E}ET], \quad (19)$$

$$P_3 = [T\bar{E}\bar{E}T]. \quad (20)$$

The sum of all pools is equal to the total concentration of active TK sites, $[E]_0$. The conformational changes between the pools are described by the following differential equations:

$$\begin{cases} \dfrac{dP_1}{dt} = \gamma_{[E\bar{E}T]}k_{-1}P_2 + \gamma_{[TE\bar{E}T]}k_{-1}P_2 - \gamma_{[EET]}k_{+1}P_1 - 2\gamma_{[TEET]}k_{+1}P_1, \\ \dfrac{dP_2}{dt} = -\gamma_{[E\bar{E}T]}k_{-1}P_2 - \gamma_{[TE\bar{E}T]}k_{-1}P_2 + \gamma_{[EET]}k_{+1}P_1 + \\ \quad + 2\gamma_{[TEET]}k_{+1}P_1 - \gamma_{[TE\bar{E}T]}k_{+2}P_2 + 2k_{-2}P_3, \\ \dfrac{dP_3}{dt} = \gamma_{[TE\bar{E}T]}k_{+2}P_2 - 2k_{-2}P_3 \ . \end{cases} \quad (21)$$

In these equations $\gamma_{[EET]}$, $\gamma_{[TEET]}$, $\gamma_{[E\bar{E}T]}$, $\gamma_{[TE\bar{E}T]}$ are the enzyme quantities in the corresponding pools:

$$\gamma_{[EET]} = \frac{2K_d[T]}{K_d^2 + 2K_d[T] + [T]^2}, \quad (22)$$

$$\gamma_{[TEET]} = \frac{[T]^2}{K_d^2 + 2K_d[T] + [T]^2}, \quad (23)$$

$$\gamma_{[E\bar{E}T]} = \frac{K_d}{K_d + [T]}, \quad (24)$$

$$\gamma_{[TE\bar{E}T]} = \frac{[T]}{K_d + [T]}. \quad (25)$$

Taking into consideration the expression for dissociation constant

$$K_d = \frac{2\gamma_{[EE]}[T]}{\gamma_{[EET]}} = \frac{\gamma_{[EET]}[T]}{2\gamma_{[TEET]}} = \frac{\gamma_{[E\bar{E}T]}[T]}{\gamma_{[TE\bar{E}T]}} \quad (26)$$

and the equations of material balance, $\gamma_{[EE]} + \gamma_{[EET]} + \gamma_{[TEET]} = 1$ and $\gamma_{[E\bar{E}T]} + \gamma_{[TE\bar{E}T]} = 1$, we will obtain the fol-

lowing formula characterizing the change in absorbance with time:

$$\frac{dA}{dt} = \varepsilon\left(\frac{dP_2}{dt} + 2\frac{dP_3}{dt}\right). \quad (27)$$

We have used Eqs. (21) and (27) for the analysis of kinetic curves for binding of ThDP to TK shown in Fig. 3a. The value of the rate constant for forward conformational change, k_{+2}, was calculated for each kinetic curve. Curve fitting was performed using the Scientist computer program. Average k_{+2} value was equal to 0.42 ± 0.01 sec^{-1}. Then, knowing the K_2 value and having determined k_{+2}, it is possible to calculate the k_{-2}: $k_{-2} = K_2 k_{+2} = 0.0028 \pm 0.0001$ sec^{-1}.

The titration curve of apoTK with thiamine diphosphate in the presence of a donor substrate, hydroxypyruvate, is shown in Fig. 4 (curve *2*). As seen from the figure, a significant part of the initial region of the titration curve is a straight line, which indicates a very high affinity of ThDP to the cofactor binding site in the first active center: all added coenzyme is completely bound by apoprotein. For this reason we were not able to determine the K_1 value (and also k_{-1}), and in all further calculations we assumed that K_1 has a rather low value equal to 10^{-4}. Based on the data shown in Fig. 4 (curve *2*), using Eqs. (9), (10), (16), and (17), assuming that K_1 is equal to 10^{-4}, and K_d equal to the K_d value in the absence of substrate (1.6 mM), we obtained the following values for ε and K_2: $\varepsilon = 0.00269 \pm 0.00002$ μM^{-1}·cm^{-1} and $K_2 = 0.008 \pm 0.001$. It should be noted that the same values for the parameters ε and K_2 were obtained also in the case when we used lower values for K_1 in our calculations (less than 10^{-4}).

After the processing of experimental data shown in the insert to Fig. 3 using Eqs. (18)-(27), the following values for kinetic rate constants of the conformational transitions in the second active site were obtained: $k_{+2} = 0.037 \pm 0.005$ sec^{-1} and $k_{-2} = 0.00030 \pm 0.00004$ sec^{-1}.

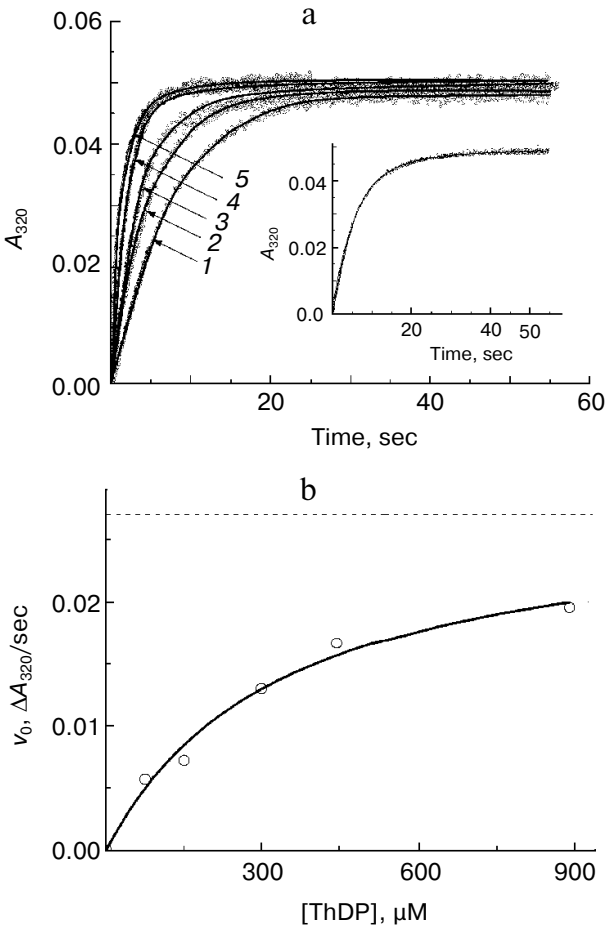


Fig. 5. Kinetics of interaction between ThDP and apotransketolase. Conditions: 25 mM glycyl-glycine buffer, pH 7.6, 2.5 mM $CaCl_2$, 6.75 μM (dimer) apoTK, temperature 25°C. a) Kinetic curves were obtained for the following ThDP concentrations: 75 (*1*), 150 (*2*), 300 (*3*), 445 (*4*), and 890 (*5*) μM. Insert shows the kinetics of ThDP binding by transketolase in the presence of 1 mM hydroxypyruvate; [ThDP] = 75 μM [26]. Solid curves were calculated based on Eqs. (18)-(27). b) Dependence of initial rate of ThDP binding with apoTK (v_0) on initial ThDP concentration. The points were obtained experimentally, and the solid curve was calculated from Eq. (14). Horizontal dashed line corresponds to limiting v_0 value at [ThDP] → ∞.

DETERMINATION OF EQUILIBRIUM AND KINETIC CONSTANTS OF INDIVIDUAL STAGES OF THE INTERACTION BETWEEN ThDP AND apoTK IN THE PRESENCE OF Ca^{2+}

Analysis of experimental data on ThDP binding by transketolase using Ca^{2+} as a cofactor was performed in the same way as in the presence of Mg^{2+}. Data on reconstruction kinetics of holoTK from apoTK and coenzyme obtained by the stopped flow method are shown in Fig. 5a. Figure 5b shows the dependence of the initial rate of the interaction between ThDP and apoTK (v_0) on the concentration of coenzyme, constructed based on Fig. 5a. Analysis of this dependence using Eq. (14) gives the following values of the parameters K_d and $v_{0,lim}$: $K_d = 340 \pm 80$ μM and $v_{0,lim} = 0.0027 \pm 0.0002$ ΔA_{320}/sec.

Data of spectrophotometric titration of apoTK by thiamine diphosphate are shown in Fig. 6 (curve *1*). As in the case of experiments with Mg^{2+} in the presence of substrate (Fig. 4, curve *2*), the affinity of coenzyme toward TK is so high that the first portions of added coenzyme completely bind to the first enzyme active site, and the initial region of the kinetic curve is a straight line. Therefore, as in the case of Mg^{2+} in the presence of substrate, it was not possible to determine the value of the equilibrium constant for the first enzyme active site, K_1 (as well as k_{-1}), and we assumed that the value of K_1 is also equal to 10^{-4}. The values of other parameters determined

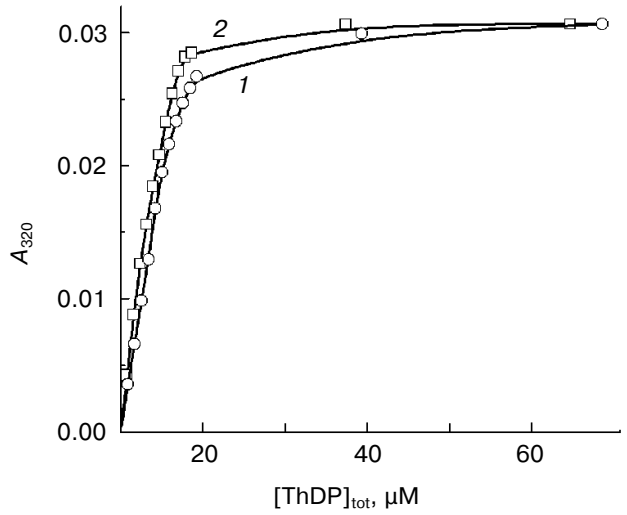


Fig. 6. Reconstruction of holoTK from apoTK and coenzyme in the absence of substrate (*1*) and presence of 2.5 mM hydroxypyruvate (*2*). Conditions: 50 mM glycyl-glycine buffer, pH 7.6, 2.5 mM $CaCl_2$, apoTK (0.67 mg/ml), temperature 25°C. The points were obtained experimentally [25], and solid curves were calculated from Eqs. (9), (10), (16), and (17).

based on the data in Figs. 5 and 6 (curve *1*) are the following: $\varepsilon = 0.00375 \pm 0.00002$ $\mu M^{-1} \cdot cm^{-1}$, $K_2 = 0.0013 \pm 0.0001$ (similar values for these parameters were obtained if lower values for K_1 were used instead of 10^{-4}); $k_{+1} = 0.8 \pm 0.1$ sec^{-1}; $k_{+2} = 0.23 \pm 0.05$ sec^{-1}; $k_{-2} = 0.00029 \pm 0.00006$ sec^{-1}.

The presence of a donor substrate during the titration of TK with thiamine diphosphate is accompanied by the increase in coenzyme affinity to apoprotein (compare curves *1* and *2* in Fig. 6). Therefore, those limitations that existed for the determination of kinetic characteristics of individual stages of the interaction between ThDP and apoTK in the absence of substrate remain, of course, also in this case. Analyzing the data shown in Fig. 6 and insert to Fig. 5 with these limitations, the following values of studied parameters were obtained: $\varepsilon = 0.00375 \pm 0.00002$ $\mu M^{-1} \cdot cm^{-1}$; $K_2 = 0.00061 \pm 0.00004$; $k_{+2} = 0.32 \pm 0.04$ sec^{-1} and $k_{-2} = 0.00019 \pm 0.00002$ sec^{-1} (the K_d value for the calculations was assumed to be equal to the one obtained in the experiments in the absence of substrate, see above).

The maximal activity of transketolase is not dependent on which of the divalent cations, Ca^{2+} or Mg^{2+}, is used as a cofactor. However, ThDP affinity to the enzyme's active sites is different: it is higher in the presence of Ca^{2+} than in the presence of Mg^{2+}. Donor substrate, but not acceptor substrate, has an effect on the rate of individual stages of the interaction between the cofactor and the protein. Especially strong effect of substrate (in the presence of Mg^{2+}) is directed on the second stage of ThDP binding to the enzyme (Scheme 1), which is accompanied by conformational changes in the protein molecule. It is interesting that the rates of forward (k_{+2} on Scheme 3) and backward (k_{-2} on Scheme 3) conformational transitions both change. These changes are the same in both cases; therefore, the equilibrium constant (K_2 on Scheme 3) determined as a ratio of kinetic constants (k_{-2} and k_{+2}) changes only slightly. This is true for the second active site of TK. In the first active site, the picture is somewhat different: equilibrium constant K_1 in the presence of a donor substrate decreases at least 6 times.

In the presence of Ca^{2+} the character of effect of donor substrate on ThDP binding in the second active site is, in principle, the same as in the presence of Mg^{2+}. The only difference is that the change in the k_{-2} value is more dramatic than in k_{+2} value, resulting in a more noticeable change in the equilibrium constant K_2.

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