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# Effect of Transketolase Substrates on Holoenzyme Reconstitution and Stability

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**Abstract**—The influence of transketolase substrates on the interaction of apotransketolase with its coenzyme thiamine diphosphate (TDP) and on the stability of the reconstituted holoenzyme was studied. Donor substrates increased the affinity of the coenzyme for transketolase, whereas acceptor substrate did not. In the presence of magnesium ions, the active centers of transketolase initially identical in TDP binding lose their equivalence in the presence of donor substrates. The stability of transketolase depended on the cation type used during its reconstitution—the holoenzyme reconstituted in the presence of calcium ions was more stable than the holoenzyme produced in the presence of magnesium ions. In the presence of donor substrate, the holoenzyme stability increased without depending on the cation used during the reconstitution. Donor substrate did not influence the interaction of apotransketolase with the inactive analog of the coenzyme N3′-pyridyl thiamine diphosphate and did not stabilize the transketolase complex with this analog. The findings suggest that the effect of the substrate on the interaction of the coenzyme with apotransketolase and on stability of the reconstituted holoenzyme is caused by generation of 2-(α,β-dihydroxyethyl)thiamine diphosphate (an intermediate product of the transketolase reaction), which has higher affinity for apotransketolase than TDP.

*Key words*: transketolase, thiamine diphosphate, 2-(α,β-dihydroxyethyl)thiamine diphosphate, spectrophotometric titration, regulation of enzyme activity

Transketolase (TK) catalyzes one of the key reactions in the pentose phosphate pathway of carbohydrate metabolism, the cleavage of keto-sugars (donor substrates) at the C–C bond adjacent to the keto-group, and also the subsequent transfer of the two-carbon fragment onto an aldose (acceptor substrate). Bivalent metal ions and thiamine diphosphate (TDP) are cofactors of TK [1]. Native TK contains 2 g·atom $Ca^{2+}$ per mole protein [2]. The properties of TK from baker's yeast are the most completely studied. The enzyme is a homodimer and has two active centers located at the interface of two contacting monomer surfaces [3, 4]. The active centers have the same enzymatic activity but differ in the affinity for TDP [5-9]. In the presence of $Ca^{2+}$, there is negative cooperativity in the binding of TDP by the active centers of TK [5-9]. In the presence of $Mg^{2+}$, the nonequivalence in behavior of the active centers with respect to TDP is either absent [10, 11] or poorly manifested [9]. X-Ray analysis fails to detect the nonequivalence of the active centers [3, 4, 12] that is observed on the functional level.

The binding of TDP with the active centers of TK is a two-step process [13]:

$$TK + TDP \xrightleftharpoons{K_d} TK^{\cdots} TDP \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} TK^{*}\text{-}TDP.$$

In the first step, which is fast and easily reversible, TDP interacts with apoTK with the involvement of the terminal pyrophosphate residue of the coenzyme, and this produces an easily dissociating intermediate inactive complex TK···TDP. Then the coenzyme binds to the protein by additional sites with the production of a sufficiently stable and catalytically active holoenzyme, TK*-TDP. The second step is slow and associated with conformational changes in the protein molecule. The two-step

*Abbreviations*: TK) transketolase; TDP) thiamine diphosphate; DHETDP) 2-(α,β-dihydroxyethyl)thiamine diphosphate.

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mechanism of TDP binding with each of two active centers of the enzyme has been analyzed using a kinetic model considering the cooperative interaction of the two initially identical active centers of apoTK [7-9]. The nonequivalence of the active centers in the coenzyme binding appeared to be caused by increase in the constant of the reverse conformational transition $k_{-1}$ of one active center relatively to the other, i.e., it was the consequence of the conformational instability of one of the active centers.

X-Ray analysis has revealed a difference in positions of two loops in each of the two subunits (residues 187-198 and 383-394), these loops being chaotic in the apoenzyme and ordered in the holoenzyme. The loops are characterized by high mobility, and they directly contact the coenzyme in holoTK [12]. It must not be ruled out that the mutually dependent counter-phase movement of these loops is responsible for the alternate destabilization of the active centers of the holoenzyme [7, 8].

A donor substrate added to holoTK is cleaved with production of a rather stable (in the absence of acceptor substrate) intermediate of the transketolase reaction, the α-carbanion 2-(α,β-dihydroxyethyl)thiamine diphosphate (DHETDP). X-Ray analysis has shown that DHETDP forms additional bonds, as compared to TDP, with amino acid residues in the active center of the enzyme and, as a result, has higher affinity for the apoprotein than the coenzyme. However, this is not accompanied by changes in the protein conformation [14].

Though the TK structure and general mechanism of thiamine catalysis are quite well studied, there are actually no data on regulation of this enzyme.

## MATERIALS AND METHODS

**Reagents.** TDP was from Serva (Germany); $CaCl_2$, hydroxypyruvate, dihydroxyacetone, xylulose 5-phosphate, ribose 5-phosphate, and glyceraldehyde were from Sigma (USA); $MgCl_2$ was from Fluka (Germany); N3′-pyridyl-TDP was synthesized as described in [15].

**Preparation of transketolase.** Apotransketolase of baker's yeast with specific activity of 20 U/mg was isolated as described in [16]. The enzyme was homogenous as shown by SDS-PAGE. The concentration of TK was determined spectrophotometrically assuming $A^{1\%}_{1\ cm} = 14.5$ at 280 nm [17]. To prepare holoTK, the enzyme solution was supplemented with bivalent metal ions at the concentration of 2.5 mM and saturating concentrations of TDP: in the presence of $Ca^{2+}$ and $Mg^{2+}$ the TDP concentration was 60 and 600 μM, respectively.

**Determination of transketolase activity.** The transketolase activity was determined spectrophotometrically at 25°C by the rate of $NAD^+$ reduction, with glyceraldehyde-3-phosphate dehydrogenase as an auxiliary enzyme [1].

**Difference absorption spectra.** Spectra of apo- and holoTK (0.5 mg/ml) in the presence and in the absence of substrate and also the spectrum of the N3′-pyridyl-TDP–TK complex were recorded with an AMINCO DW 2000 spectrophotometer (USA) in 50 mM glycyl-glycine buffer (pH 7.6) in the presence of 2.5 mM $CaCl_2$ or $MgCl_2$ and saturating concentrations of TDP or N3′-pyridyl-TDP. The difference spectra of TK in the presence of TDP and its analog were obtained by subtraction of individual spectra of apoTK and the added components.

**Spectrophotometric titration.** It has been shown [18, 19] that the reconstitution of holoTK from the apo- and coenzyme results in the appearance in the absorption spectrum of a new band that is absent in the initial components (spectrum *1* in Fig. 1). The intensity of this band is proportional to the fraction of the reconstituted catalytically active holoenzyme [19, 20]. Addition of a donor substrate increases the intensity of the induced absorption band, and its maximum slightly shifts towards shorter wavelength (spectrum *2* in Fig. 1). Spectrum *2* characterizes the absorption of the TK–DHETDP complex.

The binding of apoTK with TDP in both the absence and presence of substrates was recorded at 320 nm because the absorption of holoTK at this wavelength is still sufficiently high and is insignificantly changed on addition of donor substrate (compare *1* and *2* in Fig. 1).

HoloTK (0.54 mg/ml) was reconstituted from the apoenzyme and the coenzyme in 50 mM glycyl-glycine buffer (pH 7.6) in the presence of 2.5 mM $CaCl_2$ or $MgCl_2$. To study the effect of substrate on the reconstitution, it was introduced into the cuvette before addition of TDP. Data were recorded in two-wavelength mode ($\lambda_1$ = 320 nm and $\lambda_2$ = 400 nm) using the AMINCO DW 2000 spectrophotometer.

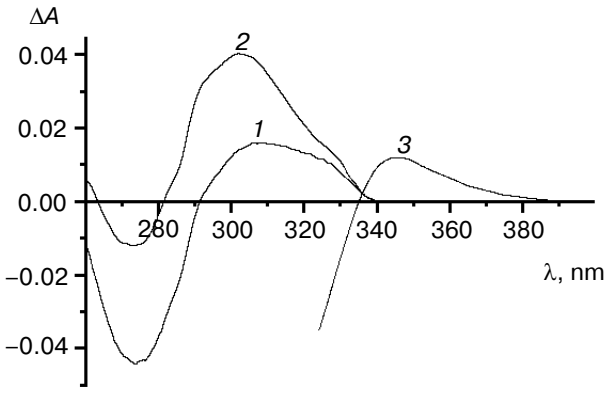


**Fig. 1.** Difference absorption spectra of TK: *1*) in the presence of TDP on subtracting the spectra of apoTK and TDP; *2*) in the presence of TDP and hydroxypyruvate on subtracting the spectra of apoTK, TDP, and hydroxypyruvate; *3*) in the presence of N3′-pyridyl-TDP on subtracting the spectra of apoTK and N3′-pyridyl-TDP. Conditions: 50 mM glycyl-glycine buffer (pH 7.6), 0.54 mg/ml TK, 2.5 mM $CaCl_2$, 0.13 mM TDP, 2.5 mM hydroxypyruvate, 65 μM N3′-pyridyl-TDP; 25°C.

A new absorption band with maximum at 345 nm was revealed in the difference absorption spectrum of the protein on the interaction of apoTK with the inactive coenzyme analog N3′-pyridyl-TDP (spectrum *3* in Fig. 1). This band was absent in the initial components. The reconstitution of the N3′-pyridyl-TDP–TK complex was followed by changes in the optical density at 345 nm in two-wavelength mode ($\lambda_1$ = 345 nm and $\lambda_2$ = 420 nm) using the AMINCO DW 2000 spectrophotometer, in 50 mM glycyl-glycine buffer (pH 7.6) in the presence of 2.5 mM $MgCl_2$. To lower the high affinity of the analog for the enzyme ($K_i$ = 1.3 nM [21]), 20 mM sodium pyrophosphate was added to the sample. To study the effect of donor substrate on the reconstitution of the N3′-pyridyl-TDP–TK complex, 2.5 mM hydroxypyruvate was added into the cuvette before the addition of the analog.

**Calculation of dissociation constants.** Based on results of the spectrophotometric titration, dissociation constants of TDP were determined for both the first and second active centers of the enzyme using Dixon's method [22]. For calculations, the Scientist program and the equation describing the interaction of the coenzyme with two initially identical active centers were used:

$$[\text{holoTK}] = \frac{0.5 \cdot [\text{TK}] \cdot [\text{TDP}_{\text{free}}]}{[\text{TDP}_{\text{free}}] + K_d^1} + \frac{0.5 \cdot [\text{TK}] \cdot [\text{TDP}_{\text{free}}]}{[\text{TDP}_{\text{free}}] + K_d^2},$$

where $K_d$ is dissociation constant.

The concentration of free TDP was determined using the formula:

$$[\text{TDP}_{\text{free}}] = [\text{TDP}_{\text{total}}] - [\text{TDP}_{\text{bound}}],$$

where $[\text{TDP}_{\text{bound}}]$ is concentration of the TK active centers that bound TDP.

**Study on the stability of holoTK and the effect of substrates.** The stability of holoTK, i.e., the ability of the enzyme to retain the bound TDP, was studied by dilution: decrease in the free cofactor concentration during dilution resulted in its dissociation from the active centers of holoTK. The amount of holoTK remaining after the dissociation was determined by its catalytic activity. The holoenzyme was previously reconstituted from apoTK (0.5-1 mg/ml) and TDP in the presence of 2.5 mM $Ca^{2+}$ or $Mg^{2+}$. Aliquots of the holoenzyme were placed into spectrophotometric cuvettes and diluted 1500-2000 times in 50 mM glycyl-glycine buffer (pH 7.6) containing 2.5 mM $Ca^{2+}$ or $Mg^{2+}$. The diluted holoenzyme was incubated for 0-60 min, then after certain time intervals the cuvettes were supplemented with the components required for determination of the transketolase activity (see above). The enzyme activity determined immediately after the dilution corresponded to the initial content of holoTK.

The effect of donor substrates on the dissociation of TDP from holoTK was studied in similar experiments. HoloTK was reconstituted in the presence of 2.5 mM $Ca^{2+}$ or $Mg^{2+}$ and of a substrate (the irreversibly cleaved donor substrate hydroxypyruvate (2.5 mM) or reversibly cleaved donor substrate dihydroxyacetone (10 mM)). Then the holoenzyme was diluted in 50 mM glycyl-glycine buffer (pH 7.6) (to 1500-2000-fold final dilution) supplemented with either 2.5 mM hydroxypyruvate or 10 mM dihydroxyacetone. Samples with the diluted enzyme were incubated for 0-60 min, and then the components required for determination of the residual activity were added.

## RESULTS AND DISCUSSION

**Effect of substrates on the reconstitution of holotransketolase.** The catalytic transformation of donor substrate under the influence of TK results in a key intermediate of the transketolase reaction, the α-carbanion DHETDP, which has higher affinity for the enzyme than TDP [6, 23]. Therefore, we supposed that a donor substrate should increase the affinity of TDP for apoTK and thus stabilize the holoenzyme. (By stabilization we mean increase in the affinity of the coenzyme for the apoprotein.)

Figure 2a presents results of spectrophotometric titration of apoTK with thiamine diphosphate in the presence of $Ca^{2+}$. Addition of hydroxypyruvate slightly increased the affinity of the coenzyme for TK. ApoTK in the presence of $Ca^{2+}$ is characterized by a relatively high affinity for TDP: all coenzyme added into the sample is stoichiometrically bound to apoTK to 50% saturation (i.e., in the first active center). Therefore, it was impossible to study in such an experiment the donor substrate effect on the TDP binding in the first active center. The dissociation constant of TDP for the second active center was calculated, and in the presence of substrate the value of $K_d^2$ was 3.6-fold decreased and became 0.21 μM, whereas in the absence of the substrate its value was 0.76 μM (table).

Experiments in the presence of $Mg^{2+}$ (Fig. 2b) were more informative, because in this case the affinity of TDP for apoTK was significantly lower than in the presence of $Ca^{2+}$ [5, 6, 9-11]. Spectrophotometric titration showed that the affinity of the coenzyme for both active centers in the presence of $Mg^{2+}$ is the same (curve *1*): $K_d^1 = K_d^2 = 5.2$ μM (table). On addition of the donor substrate, the affinities of the enzyme active centers for TDP increased, but differently. And in the presence of the irreversibly cleaved donor substrate (hydroxypyruvate) (curve *2*), the affinity of TDP for apoTK in the first active

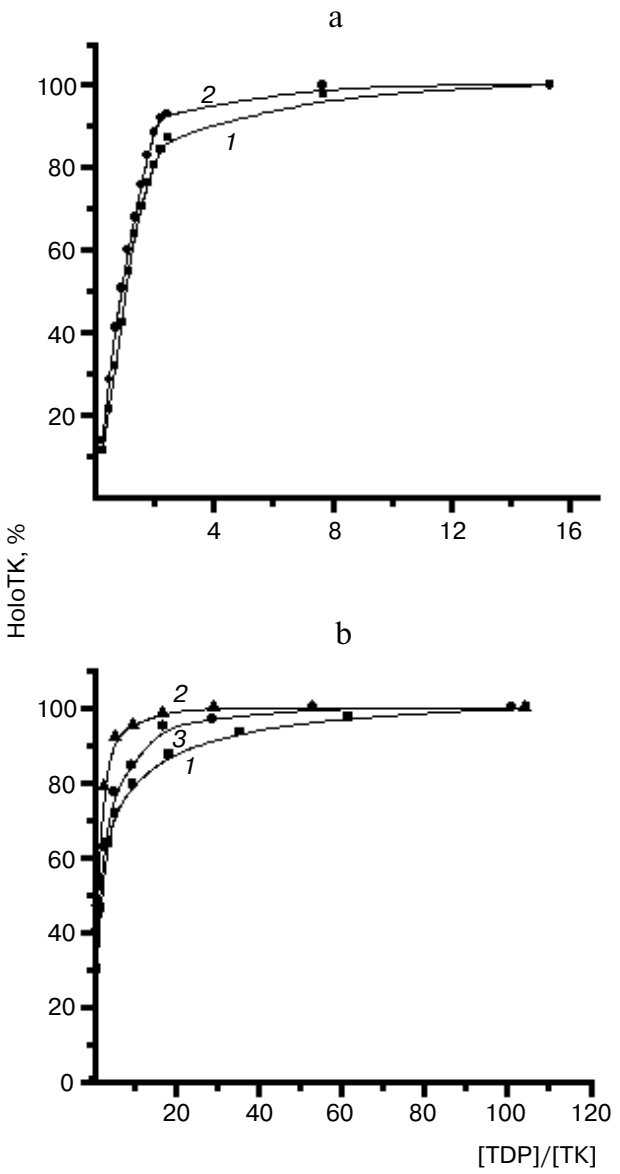


**Fig. 2.** Reconstitution of holoTK from apoTK and the coenzyme in the presence of calcium (a) or magnesium (b): *1*) without substrate; *2*, *3*) in the presence of 2.5 mM hydroxypyruvate or 10 mM dihydroxyacetone, respectively. Medium composition: 50 mM glycyl-glycine buffer (pH 7.6), 2.5 mM $CaCl_2$ or $MgCl_2$, 0.67 mg/ml TK; 25°C.

center increased so strongly that it was impossible to determine the value of $K_d^1$. The value of $K_d^2$ for the second active center was decreased less and became 1.6 μM (table).

A similar although less pronounced effect was observed in the presence of the reversibly cleaved donor substrate (dihydroxyacetone) (curve *3*): $K_d^1$ = 0.16 μM, $K_d^2$ = 5.2 μM. Note that unlike the experiments with hydroxypyruvate, only the value of $K_d^1$ was decreased, whereas the value of $K_d^2$ was unchanged (table). Thus, in the presence of both $Ca^{2+}$ and $Mg^{2+}$, donor substrates not only increased the affinity of TDP for apoTK but also induced negative cooperativity in the coenzyme binding, which was absent when no substrate was added.

We suppose that the effect of the donor substrate on the affinity of apoTK for TDP depends on generation of the intermediate of the transketolase reaction, the α-carbanion DHETDP, which has higher affinity for the enzyme than TDP. This would be confirmed by the absence of the acceptor substrate effect on the interaction of TDP with apoTK. In fact, the acceptor substrate glyceraldehyde had no effect on the amount of holoenzyme formed (table).

The absence of the donor substrate effect on the interaction of apoTK with the inactive analog of TDP, N3′-pyridyl-TDP, also confirmed our hypothesis. This analog lacks the nitrogen atom in the first position of the aminopyridine ring, and this robs it of ability for producing a hydrogen bond with Glu418, which is required for catalysis [16]. With respect to TDP, N3′-pyridyl-TDP is a competitive inhibitor of TK with a very high affinity for it ($K_i$ = 1.3 nM [21]).

The binding of N3′-pyridyl-TDP with the active centers of TK is associated with appearance of a new absorption band in the same region of the circular dichroism spectrum as in the case of interaction of the native coenzyme [24, 25]. X-Ray analysis of the N3′-pyridyl-TDP complex with TK has shown that on incorporation into the active center of the enzyme the analog acquires a V-conformation specific for TDP bound to the enzyme [26]. These data indicated that the binding of this analog with TK and the microenvironment in the active center were adequate and the same as in the case of TDP. The

Constants of dissociation of TDP for the two active centers of TK

| Experimental conditions | $K_d^1$, μM | $K_d^2$, μM |
|---|---|---|
| $Ca^{2+}$ | | |
| without substrate | * | 0.76 |
| 2.5 mM hydroxypyruvate | * | 0.21** |
| $Mg^{2+}$ | | |
| without substrate | 5.2 | 5.2 |
| 2.5 mM hydroxypyruvate | * | 1.6** |
| 10 mM dihydroxyacetone | 0.16** | 5.2** |
| 10 mM glyceraldehyde | 5.4 | 5.4 |

* In experiments with calcium or magnesium the affinity of TDP for TK in the presence of hydroxypyruvate was so high that it was impossible to determine $K_d$ for the first active center.

** In this case the $K_d$ value was apparent, because the determination was performed in the presence of donor substrate.

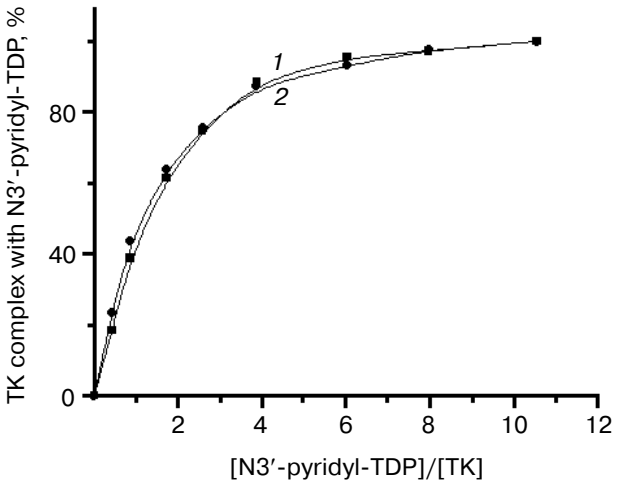


**Fig. 3.** Reconstitution of the N3′-pyridyl-TDP–TK complex from apoTK and N3′-pyridyl-TDP: *1*) in the absence of substrate; *2*) in the presence of 2.5 mM hydroxypyruvate. Medium composition: 50 mM glycyl-glycine buffer (pH 7.6), 0.5 mg/ml TK, 2.5 mM $MgCl_2$, and N3′-pyridyl-TDP (1-36.6 μM); 25°C. To lower the high affinity of N3′-pyridyl-TDP to the enzyme, 20 mM sodium pyrophosphate was added into the samples (see "Materials and Methods").

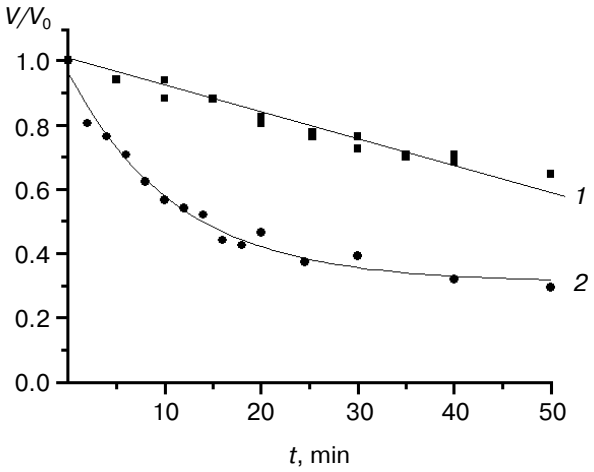


**Fig. 4.** Stability of holoTK reconstituted in the presence of $Ca^{2+}$ (*1*) or $Mg^{2+}$ (*2*). Incubation medium: 50 mM glycyl-glycine buffer (pH 7.6), 1 μg/ml holoTK, 2.5 mM $Me^{2+}$; 25°C.

difference absorption spectrum of the N3′-pyridyl-TDP complex with TK (Fig. 1, spectrum *3*) was characterized by an induced absorption band with maximum at 345 nm, and this allowed us to record the interaction of N3′-pyridyl-TDP with apoTK by spectrophotometric titration.

To decrease the high affinity of apoTK for N3′-pyridyl-TDP, sodium pyrophosphate (which is a competitive inhibitor of TK relative to TDP, and, respectively, to the analog) was added into the sample [13]. Figure 3 presents results of spectrophotometric titration of apoTK with the analog in the presence of 20 mM sodium pyrophosphate. Curve *1* shows the generation of the inactive N3′-pyridyl-TDP complex with TK in the absence of substrate, and curve *2* shows the same in the presence of 2.5 mM hydroxypyruvate. The virtual coincidence of these curves indicates that the donor substrate has no effect on the affinity of the inactive coenzyme analog for apoTK.

Summarizing the data on the interactions of TDP and its inactive analog with apoTK and on the effect of donor substrate (ketose) on these interactions, we conclude that the donor substrate displays the effect only when the active holoenzyme is generated and has no effect on generation of the inactive N3′-pyridyl-TDP–TK complex. This confirms our hypothesis that the increase in the affinity of TDP for apoTK in the presence of donor substrate is caused by production of the intermediate α-carbanion DHETDP during the transketolase reaction.

**Effect of donor substrate on stability of holoTK.** The binding of apoTK with TDP is reversible, and with decrease in the concentration of free TDP in the medium, e.g., on dilution, TDP dissociates from the active centers of holoTK. Experimental data on effects of donor substrates on the stability of holoTK reconstituted under varied conditions are shown below. Stability means the affinity of TDP for apoTK. This affinity was evaluated by the transketolase activity, which corresponded to the amount of the undissociated holoenzyme.

Figure 4 presents the decrease in the holoTK activity after the enzyme dilution depending on the cation ($Ca^{2+}$ or $Mg^{2+}$) used in the holoenzyme reconstitution. Curve *1* shows the decrease in the activity depending on the incubation time of the diluted holoenzyme reconstituted in the presence of $Ca^{2+}$: incubation for 50 min decreased the enzyme activity by ~40%. Thus, TDP dissociated only from the second active center, which in the presence of $Ca^{2+}$ displayed the lower affinity for the coenzyme than the first center: the constant values have about one order of magnitude difference [5-9].

The holoenzyme reconstituted in the presence of $Mg^{2+}$ was less stable than the enzyme reconstituted in the presence of $Ca^{2+}$: incubation of the diluted holoenzyme for 50 min resulted in 70% decrease in its activity (curve *2* in Fig. 4). Thus, in this case TDP dissociated during the incubation from both active centers. These facts correspond to the lower affinity of apoTK for TDP in the presence of $Mg^{2+}$ than in the presence of $Ca^{2+}$ [5, 6, 9].

The effects of donor substrates, the irreversibly cleaved hydroxypyruvate and the reversibly cleaved dihydroxyacetone, on the stability of holoTK reconstituted in the presence of $Ca^{2+}$ are shown in Fig. 5a. Both hydroxypyruvate and dihydroxyacetone decreased the degree of TDP dissociation from holoTK as compared to the control in the absence of the substrate. And, as expected, the

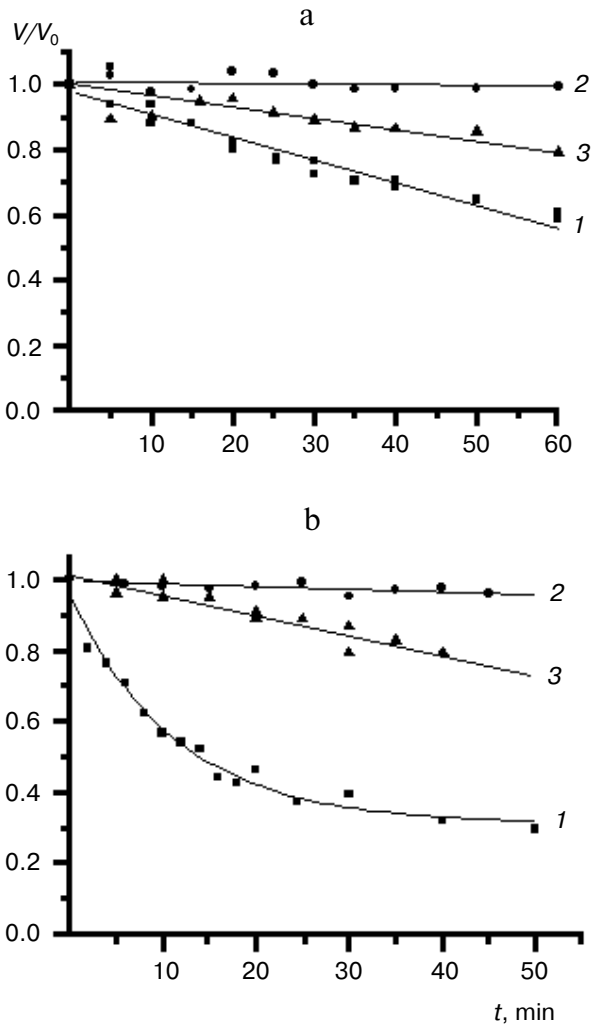


**Fig. 5.** Effects of donor substrates on the stability of holoTK reconstituted in the presence of calcium (a) or magnesium (b): *1*) without substrate; *2*, *3*) in the presence of 2.5 mM hydroxypyruvate or 10 mM dihydroxyacetone, respectively. Incubation medium: 50 mM glycyl-glycine buffer (pH 7.6), 1 μg/ml holoTK, 2.5 mM $Ca^{2+}$ or $Mg^{2+}$; 25°C.

effect of hydroxypyruvate was more pronounced than the effect of dihydroxyacetone.

The effects of donor substrates on stability of holoTK reconstituted in the presence of $Mg^{2+}$ are shown in Fig. 5b. As in the experiments with $Ca^{2+}$, the donor substrates stabilized holoTK. And the final effects were virtually the same as the effect observed in experiments in the presence of $Ca^{2+}$. In other words, stability of holotransketolase in the presence of donor substrate virtually does not depend on the cation used during its reconstitution.

The concept that the donor substrate function responsible for stabilization of holoTK is caused by generation of α-carbanion DHETDP, which is an intermediate of the transketolase reaction, is supported by experiments which, first, have shown that the acceptor substrate has no effect on the degree of holoTK dissociation to apoTK and the coenzyme and, second, that the donor substrate has no effect on stability of the apoTK complex with the inactive analog of the coenzyme, N3′-pyridyl-TDP (data not presented).

Based on the experimental findings presented in this paper, it is suggested that the effect of donor substrate on the interaction of TK with the coenzyme at its low concentration should be a regulatory mechanism of activity of this enzyme; it seems that this mechanism is common for all thiamine diphosphate-dependent enzymes. This viewpoint is supported by the literature [6, 27-30].

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