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# Kinetic study of the H103A mutant yeast transketolase

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**Abstract Data from site-directed mutagenesis and X-ray crystallography show that His103 of holotransketolase (holoTK) does not come into contact with thiamin diphosphate (ThDP) but stabilizes the transketolase (TK) reaction intermediate, ,-dihydroxyethyl-thiamin diphosphate, by forming a hydrogen bond with the oxygen of its -hydroxyethyl group [Eur. J. Biochem. 233 (1995) 750; Proc. Natl. Acad. Sci. USA 99 (2002) 591]. We studied the influence of His103 mutation on ThDP-binding and enzymatic activity. It was found that mutation does not affect the affinity of the coenzyme to apotransketolase (apoTK) in the presence of $Ca^{2+}$ (a cation found in the native holoenzyme) but changes all the kinetic parameters of the ThDP–apoTK interaction in the presence of $Mg^{2+}$ (a cation commonly used in ThDP-dependent enzymes studies). It was concluded that the structures of TK active centers formed in the presence of $Mg^{2+}$ and $Ca^{2+}$ are not identical. Mutation of His103 led to a significant acceleration of the one-substrate reaction but a slow down of the two-substrate reaction so that the rates of both types of catalysis became equal. Our results provide evidence for the intermediate-stabilizing function of His103.**



*Keywords:* Thiamin diphosphate; Transketolase; Kinetic model; Kinetic constant; Induced optical activity; Mutagenesis

## 1. Introduction

Transketolase (TK), a thiamin diphosphate (ThDP)-dependent enzyme (EC 2.2.1.1), catalyzes the cleavage of a carbon–carbon bond of a ketose, donor substrate, and reversible transfer of its two-carbon unit (α,β-dihydroxyethyl group) to an aldose, acceptor substrate [3]. TK from *Saccharomyces cerevisiae* is composed from two identical subunits with a molecular mass of 74.2 kDa per monomer and it has two active centers [4–6]. TK was the first ThDP-dependent enzyme to have its crystal structure solved. The interactions of the non-covalently bound cofactor, ThDP, with the protein were identified [6,7] (Fig. 1) and very similar ThDP surrounding was found later in all enzymes of this class.

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*Abbreviations:* TK, transketolase; ThDP, thiamin diphosphate; DHEThDP, α,β-dihydroxyethyl-thiamin diphosphate; X5P, D-xylulose 5-phosphate; R5P, D-ribose 5-phosphate; GAPDH, D-glyceraldehyde 3-phosphate dehydrogenase

Each of the two identical enzyme's subunits consists of three domains: the N-terminal or PP-binding domain; the middle or pyrimidine-binding domain; and the C-terminal domain. The coenzyme is bound in a deep cleft between the two subunits, so that one subunit provides the pyrimidine-binding site and the other – the PP-binding site, while the thiazolium ring interacts with the amino acid residues of the both subunits. The aminopyrimidine ring is located in the hydrophobic pocket formed mainly by the aromatic amino acid residues of the pyrimidine-binding domain. The pyrophosphate group of ThDP is bound to the PP-binding domain directly through hydrogen bonds with its amino acid residues, and indirectly through a metal ion [7] (Fig. 1).

All ThDP-dependent enzymes, including TK, require divalent metal ions for their activity. Studies of these enzymes are commonly carried out using $Mg^{2+}$. In the native holotransketolase (holoTK), only $Ca^{2+}$ (2 g atom per 1 mole of protein) was found. The presence of divalent cations is an essential requirement for the binding of ThDP to the apoenzyme. ThDP-apotransketolase (apoTK) binding requires at least two steps [8–10] (Scheme 1).

The first step, fast and easily reversible, yields an intermediate: a catalytically inactive, primary TK···ThDP complex. The second step is slow and is accompanied by conformational changes necessary for the formation of the catalytically active holoenzyme, TK*–ThDP. The initially identical TK active centers become non-equivalent in the course of ThDP binding [11–13]. In the presence of $Mg^{2+}$, the values of the apparent ThDP-dissociation constants for the two active centers of TK differ by about three times [13], while in the presence of $Ca^{2+}$ they differ by an order of magnitude [11,12]. Affinity of both TK active centers towards ThDP is higher in presence of $Ca^{2+}$ than in the presence of $Mg^{2+}$, but with either cation ThDP binding shows the negative cooperativity, albeit with $Mg^{2+}$ it is less pronounced [13].

Replacement of His103 with alanine in TK lowered the enzyme's affinity for ThDP in the presence of $Mg^{2+}$ by about eight times [1]; although according to the X-ray data His103 does not come in direct contact with ThDP [7] (Fig. 1).

TK, being a typical transferase, is able to catalyze the so-called one-substrate reaction where only the donor substrate is utilized in the absence of the acceptor substrate [14–16]. As for the two-substrate reaction, the formation of the intermediate product, α,β-dihydroxyethyl-thiamin diphosphate (DHE-ThDP), (i.e., glycolaldehyde attached to ThDP, the so-called "active glycolaldehyde") is registered. The primary products of the one-substrate reaction with D-xylulose 5-phosphate (X5P) are D-glyceraldehyde 3-phosphate and glycolaldehyde. Free

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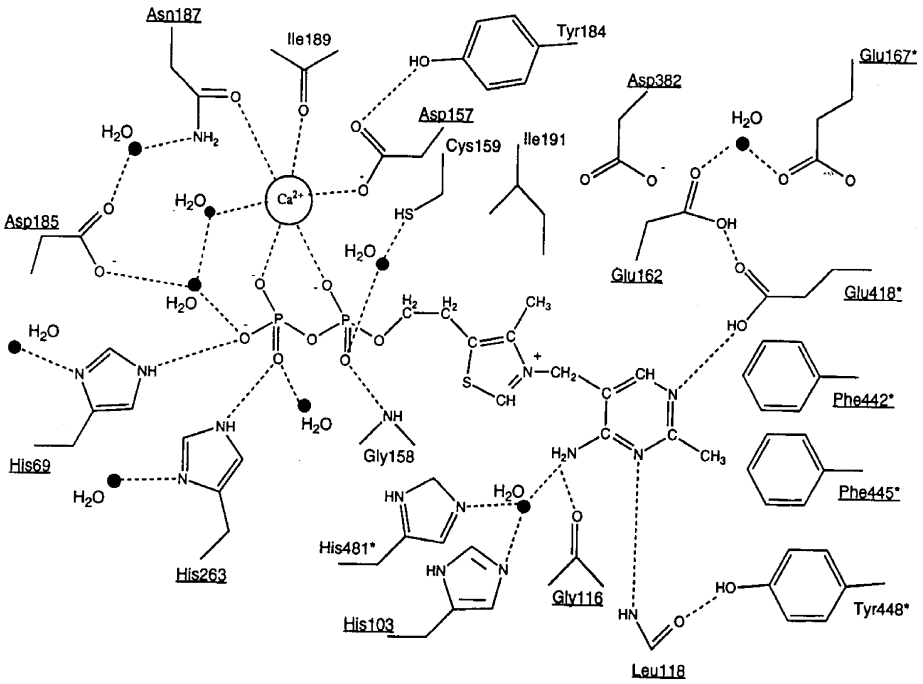



Fig. 1. Cofactor-protein interactions in the ThDP binding site of TK. Conserved residues are underlined and residues from the second subunit are marked by * [7].

$$\text{TK + ThDP} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{TK}\cdots\text{ThDP} \rightleftharpoons \text{TK*-ThDP}$$

Scheme 1. Coenzyme binding to apoTK.

glycolaldehyde, i.e., glycolaldehyde dissociated from the active center, condenses immediately with the enzyme-bound "active glycolaldehyde" formed from another molecule of X5P. Thus, the concentration of free glycolaldehyde in the reaction medium is below detectable limit. In the course of this reaction instead of glycolaldehyde erythrulose is generated. The rate of the one-substrate reaction is limited by the rate of glycolaldehyde release from DHEThDP, i.e., by stability of the intermediate. It is believed [1,2] that His103 is directly involved in DHEThDP stabilization.

The present study was undertaken to elucidate how TK mutation at His103 affects the enzyme's interaction with ThDP and its catalytic properties.

## 2. Materials and methods

### 2.1. Materials

X5P, D-ribose 5-phosphate (R5P), D-glyceraldehyde 3-phosphate dehydrogenase (GAPDH), ThDP and $CaCl_2$ were purchased from Sigma Chemical Co; $MgCl_2$ was purchased from Fluka Chemical Co; glycyl-glycine, Tris–HCl and dithiothreitol were purchased from Serva Electrophoresis; $NAD^+$ was obtained from ICN.

ThDP concentration was determined spectrophotometrically at 272.5 nm using a molar extinction coefficient of 7800 [17].

### 2.2. Enzymes preparation

The wild type TK (WT TK) and the H103A mutant TK were expressed and purified as described by Wikner et al. [18]. Both WT and H103A TK were obtained as the apoenzyme. The crystalline enzymes were stored at 4 °C in 50% saturated ammonium sulfate, pH 7.6. The enzymes were homogeneous as judged by SDS–PAGE. TK concentration was determined spectrophotometrically using $A^{1\%}_{1cm} = 14.5$ at 280 nm [19].

### 2.3. TK activity determination

The enzyme's catalytic activity in the common two-substrate reaction was measured spectrophotometrically (with an Aminco DW-2000 spectrophotometer) at 25 °C by the rate of $NAD^+$ reduction in a coupled system with GAPDH [3]. The reaction mixture (final volume, 1 ml) contained: 50 mM glycyl-glycine, 1 mM sodium arsenate, 0.37 mM $NAD^+$, 3 U GAPDH, 3.2 mM dithiothreitol, 2.5 mM $CaCl_2$, 0.08 mM ThDP, 0.45 mM X5P, 0.9 mM R5P; pH 7.6. The reaction was initiated by the addition of TK.

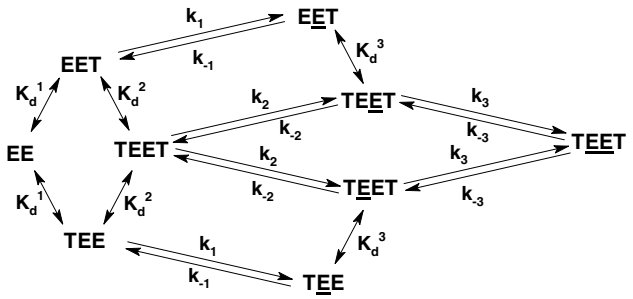
The TK activity in the one-substrate reaction was measured in the same manner but with ketose (X5P, 2.2 mM) as the donor substrate, without adding R5P, the acceptor substrate.

### 2.4. Spectrophotometric titration

ThDP binding to apoTK results in the appearance of a new band in the absorption spectrum with a wide maximum 315–320 nm [20]. The intensity of this band is proportional to the amount of the reconstituted holoenzyme [21]. Thus the kinetic of holoenzyme reconstitution was monitored by the increase in $A_{315\ nm}$, starting immediately after the addition of ThDP until equilibrium was reached. The final $A_{315\ nm}$ value at the saturating coenzyme concentration was used as the 100% reference. $A_{315\ nm}$ was recorded with an Aminco DW-2000 spectrophotometer operated in a two-wavelength mode (the reference wavelength was 360 nm). Reconstitution was carried out in 10 mM Tris–HCl buffer, pH 7.35, containing 2 mM $CaCl_2$ or $MgCl_2$. Kinetic parameters were determined as previously described based on the agreement between the calculated and experimental curves in accordance with an algorithm reported earlier [11–13].

### 2.5. Kinetic model and calculation

Regarding the reconstitution of the enzyme with two active centers, we have considered the interaction of each active center with ThDP as shown in Scheme 2. We also admit the possibility of cooperativity between the centers, i.e., the dependence of the equilibrium and rate constants of one center on the state of the other. Details of the model and the principle of the calculation are given in [11–13].




Scheme 2. ThDP interaction with apoTK.

E is the apoenzyme's active center;
T is the ThDP;
ET is the primary, readily dissociating, catalytically inactive complex of apoTK with ThDP;
ET and TEET are the holoenzymes, catalytically active at one and both active centers, respectively (only these species display the characteristic absorption band at 315 nm);
$K_d^1$, $K_d^2$ and $K_d^3 = K_d^2 k_1 k_{-2}/k_{-1}k_2$ are the dissociation constants for the primary apoTK complexes with ThDP;
$k_i$ and $k_{-i}$ ($i = 1,2,3$) are the rate constants of the forward and reverse conformational transition reactions for the different enzyme species.

## 3. Results and discussion

### 3.1. Interaction of H103A mutant apoTK with ThDP

Interaction of the H103A mutant of apoTK with ThDP was investigated by the kinetic modelling method that had previously been used in studies on WT apoTK reconstitution with ThDP [11–13]. The holoenzyme generation was monitored by changes in optical density at 315 nm.

The dependence of the equilibrium concentrations of WT and H103A holoTK on the amount of ThDP added in the presence of $Mg^{2+}$ is shown in Fig. 2 (curves 1 and 2, respectively). The affinity of H103A apoTK for ThDP in the presence of $Mg^{2+}$ is much lower than for the WT enzyme, in agreement with earlier reports [1]. The apparent $K_d$ of WT TK for both centers (0.86 and 2.2 μM) are significantly smaller than that of H103A mutant TK (18 μM).

To characterise the kinetic parameters of the individual steps of the holoTK reconstitution from the apoenzyme and coenzyme, the reconstitution kinetics of both WT and H103A TK species were investigated in the presence of $Mg^{2+}$ at different ThDP concentrations and analysed using the set of equations describing Scheme 2. The results are presented in Fig. 3A and B for WT and H103A TK, respectively. The points represent the experimental data while lines are theoretical fits. The parameters of Scheme 2, optimised by the fitting procedure, are shown in Table 1. Good correspondence of the experimental points and theoretical fits in a wide concentration range is indicative of the correctness of the selected parameters. Comparison of the primary dissociation constants ($K_d^1 = K_d^2 = K_d^3$, Scheme 2) for the WT and H103A TK species in the presence of $Mg^{2+}$ shows that in the case of mutant TK they are somewhat lower. Besides, the mutation leads to changes of all the parameters characterizing the secondary binding (Table 1). The directionality of these changes suggests the lowering of ThDPs affinity to mutant TK.

Fig. 4 shows ThDP saturation kinetic for WT (1) and H103A (2) TK in the presence of $Ca^{2+}$ at equilibrium conditions. It is

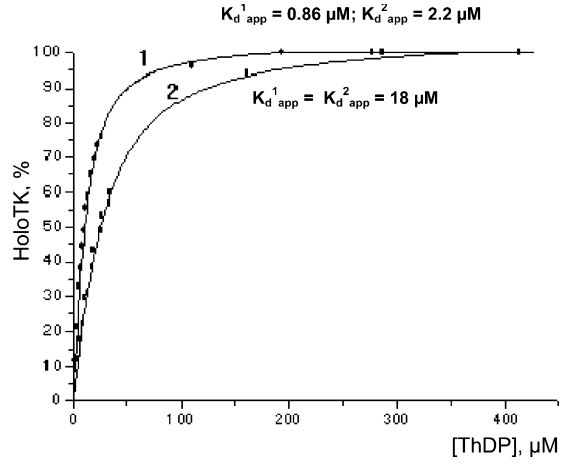



Fig. 2. Equilibrium formation of WT (curve 1) and H103A mutant (curve 2) holoTK as a function of total ThDP concentration in the presence of $Mg^{2+}$. Symbols represent experimental data point; the lines (curve fit) are calculated for a series of kinetic parameters, presented in Table 1. ApoTK (dimer), 5.9 μM (curve 1) and 6.73 μM (curve 2); $MgCl_2$, 2 mM.

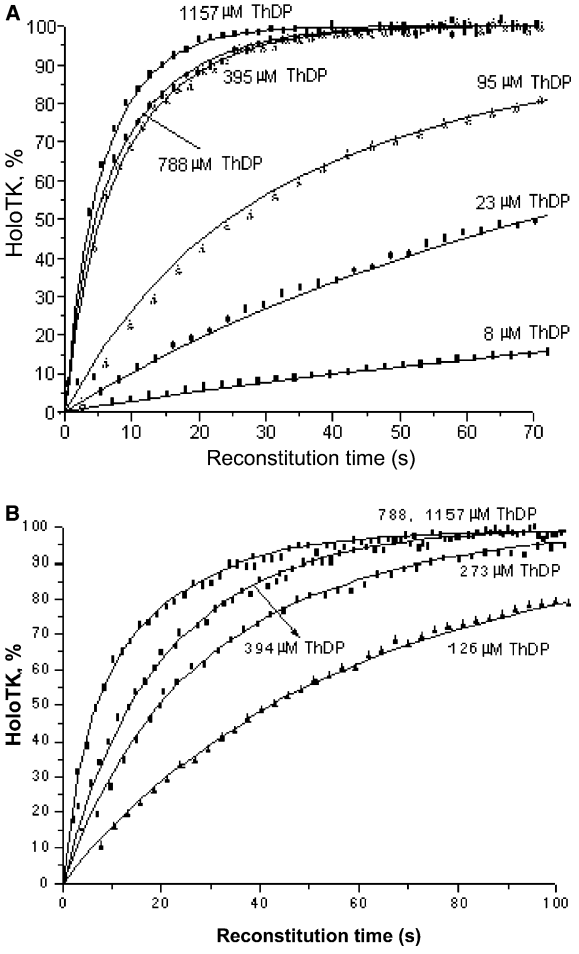



Fig. 3. Kinetics of holoTK formation at varying concentrations of once-added ThDP to WT (A) and H103A mutant (B) apoTK in the presence of $Mg^{2+}$. Symbols represent experimental data point; the lines (curve fit) are calculated for a series of kinetic parameters presented in Table 1. ApoTK (dimer), 4.76 μM (A) or 6.73 μM (B); $MgCl_2$, 2 mM.

Table 1
Kinetic parameters of WT and H103A mutant holoTK reconstitution from apoTK and ThDP in the presence of $Mg^{2+}$ or $Ca^{2+}$ (the experimental error upon determination of the above parameters is within 10–15%)

| Kinetic parameters | WT | | H103A mutant | |
|---|---|---|---|---|
| | $Mg^{2+}$ | $Ca^{2+}$ | $Mg^{2+}$ | $Ca^{2+}$ |
| *Primary binding* | | | | |
| $K_d^1$, μM | 500 | 300 | 600 | 300 |
| $K_d^2$, μM | 500 | 300 | 600 | 300 |
| $K_d^3$, μM | 500 | 300 | 600 | 300 |
| *Secondary binding* | | | | |
| $k_1$, $s^{-1}$ | 0.3 | 0.7 | 0.12 | 0.7 |
| $k_{-1}$, $s^{-1}$ | 0.002 | 0.00035 | 0.005 | 0.00035 |
| $k_2$, $s^{-1}$ | 0.3 | 0.7 | 0.12 | 0.7 |
| $k_{-2}$, $s^{-1}$ | 0.002 | 0.00035 | 0.005 | 0.00035 |
| $k_3$, $s^{-1}$ | 0.3 | 0.7 | 0.12 | 0.7 |
| $k_{-3}$, $s^{-1}$ | 0.005 | 0.003 | 0.015 | 0.003 |

clearly seen that in both cases the dependences are virtually identical. Initially, up until about 50% saturation, linearity indicates stoichiometric binding: all the ThDP added appears to be fully bound. At higher ThDP concentration a detectable fraction of the coenzyme remains unbound due to its lower affinity to the second active center. Both titration curves reach equilibrium at the added ThDP concentration of about 60 μM.

In the presence of $Ca^{2+}$, the apparent $K_d$ value for the second active center of TK was estimated as 0.33 μM for both WT and H103A TK (based on the Scheme 1 and data presented in Fig. 4). The affinity of the TKs first active center for ThDP in the presence of $Ca^{2+}$ could not be estimated by the method employed herein because the affinity was too high: all the ThDP added to the sample was stoichiometrically bound to the first active center. The $K_d$ value determined for second center in presence of $Ca^{2+}$ (0.33 μM) is smaller than the apparent $K_d$ values for both WT TK centers in presence of $Mg^{2+}$ (0.86 and 2.2 μM, see above). This result is in full agreement with the previously reported difference of TK affinity for ThDP in the presence of different cations: using $Mg^{2+}$ instead of $Ca^{2+}$ probably impedes the binding of the diphosphate group of ThDP to the protein.

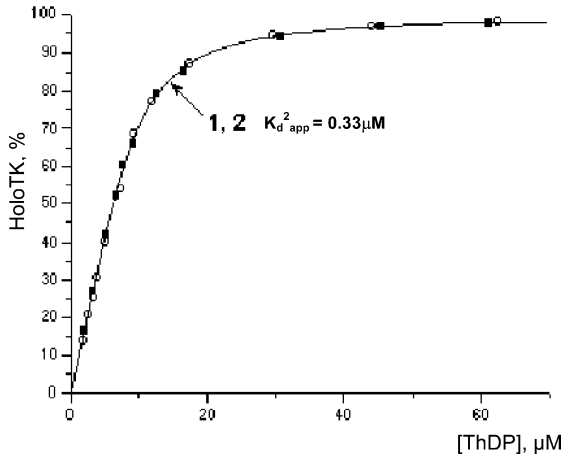



Fig. 4. Equilibrium formation of WT (1) and H103A mutant (2) holoTK as a function of total ThDP concentration in the presence of $Ca^{2+}$. Symbols represent experimental data point; the line (curve fit) is calculated for a series of kinetic parameters, presented in Table 1. WT apoTK (dimer), 14 μM; H103A mutant apoTK (dimer), 5.47 μM; $CaCl_2$, 2 mM.

To measure the individual kinetic and dissociation constants for the reconstitution of the holoTK in the presence of $Ca^{2+}$, the kinetics of interaction of the coenzyme with WT and H103A apoTK were obtained using a wide ThDP concentration range (Fig. 5A and B, respectively). The best fit of the calculated curves to the experimental data was obtained with the set of parameters presented in Table 1. Comparison of the data on WT TK and H103A TK shows that the values of all kinetic parameters characterizing the reconstitution of the holoenzyme in the presence of $Ca^{2+}$ are identical for both TK species.

Thus the replacement of His103 with alanine had no effect on coenzyme binding to apoTK in the presence of $Ca^{2+}$. This result is in agreement with the X-ray crystallography data and indicates the lack of difference in the structures of WT and H103A holoTK upon their reconstitution in the presence of $Ca^{2+}$ [1]. Yet, in the presence of $Mg^{2+}$, the mutation causes essential changes in kinetic parameters for the conformational binding stage (Table 1) leading to further destabilisation of the TK complex with ThDP.

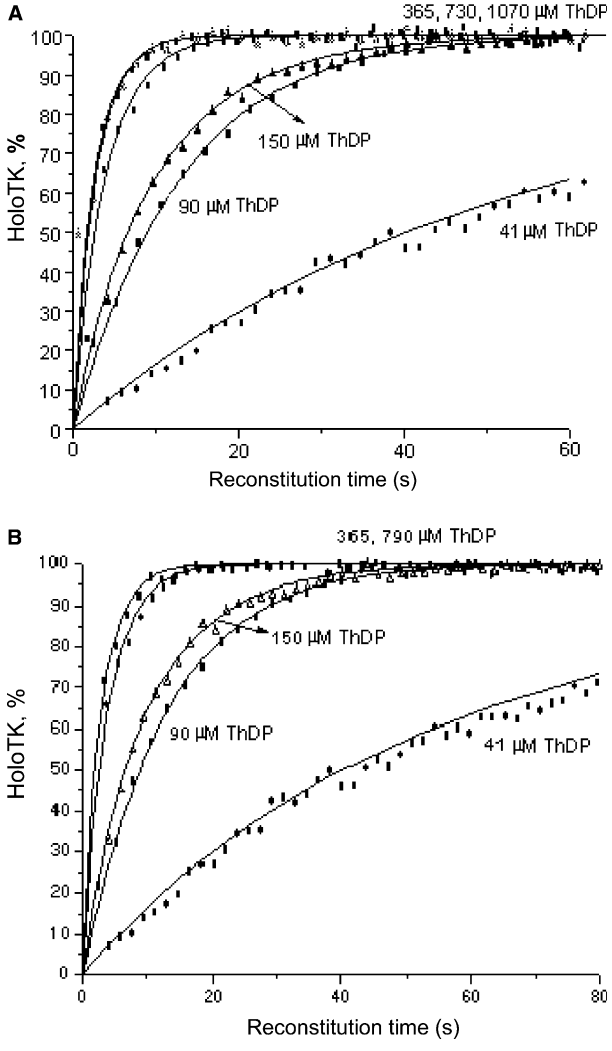



Fig. 5. Kinetics of H103A mutant holoTK formation at varying concentrations of ThDP to WT (A) and H103A mutant (B) apoTK in the presence of $Ca^{2+}$. Symbols represent experimental data point; the lines (curve fit) are calculated for a series of kinetic parameters presented in Table 1. ApoTK (dimer), 4 μM (A) and 5.47 μM (B); $CaCl_2$, 2 mM.

According to the data obtained by X-ray analysis, His103 does not come in direct contact with ThDP but appears to interact with the 4′-$NH_2$-group of its pyrimidine ring through a water molecule [7] (Fig. 1). This water molecule is part of a network of hydrogen bonds, connecting together the side chains of His103, His481 and the 4′-$NH_2$-group of ThDP. This network probably contributes to the stabilisation of the holoTK structure and must be especially important in the presence of $Mg^{2+}$, when holoTK structure appears to be less stable than in presence of $Ca^{2+}$. Replacement of His103 with alanine breaks the network of hydrogen bonds in the 4′-$NH_2$-area thereby causing the local changes in the protein's conformation and decreasing in this way the affinity of the H103A TK (compared to WT TK) for ThDP in the presence of $Mg^{2+}$. If the holoenzyme reconstitution occurs in the presence of $Ca^{2+}$, the binding of ThDP to the protein is much tighter. Therefore, local structural changes that may occur in the 4′-$NH_2$-area due to H103A substitution do not seriously disturb the apoTK–ThDP interaction in presence of $Ca^{2+}$.

The data presented herein suggests that the structure of the holoTK active center, formed in the presence of $Mg^{2+}$, is not identical to the one formed in the presence of $Ca^{2+}$.

### 3.2. Influence of His103 mutation on the catalytic properties of TK

Replacement of His103 with alanine does not deprive TK of its catalytic activity but markedly influences its quantitative characteristics. The rate of the two-substrate TK reaction (line 3, Fig. 6) is essentially decreased compared to the WT TK-catalyzed reaction (line 1, Fig. 6). The opposite is true for the one-substrate reaction: the mutation increased the rate sevenfold (compare lines 4 and 2, Fig. 6) before becoming equal to the rate of the two-substrate reaction (line 3, Fig. 6).

His103 is located at the bottom of the substrate channel. The X-ray analysis of the TK-reaction intermediate, DHEThDP, in the TK active center suggests that His103 directly participates in the stabilization of the intermediate by forming a hydrogen bond with the oxygen of its β-hydroxyl group [2]. Note, that the rate of one-substrate reaction is limited by the rate of glycolaldehyde release from DHEThDP [14,15] (i.e., by the stability of the "active glycolaldehyde" intermediate formed during binding and splitting of the donor substrate).

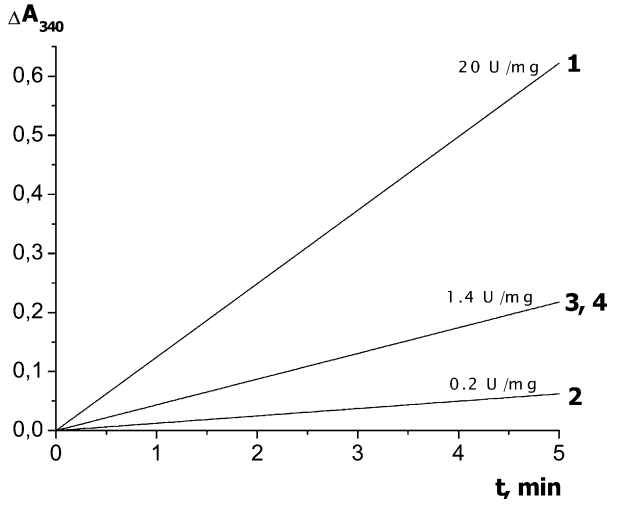



Fig. 6. Determination of TK activity by changes in optical density at 340 nm. Two-substrate reaction catalyzed by WT and H103A mutant TK, respectively, (1, 3); one-substrate reaction catalyzed by WT and H103A mutant TK, respectively, (2, 4). Reaction mixture (final volume 1 ml) contained: 50 mM glycyl-glycine, 1 mM sodium arsenate, 0.37 mM $NAD^+$, 3 U GAPDH, 3.2 mM dithiothreitol, 2.5 mM $CaCI_2$, 80 μM ThDP, 1 μg WT TK (1), 10 μg WT TK (2) and 5 μg H103A mutant TK (3, 4), respectively; 900 μM R5P, and 450 μM (1, 3), 2200 μM (2, 4) X5P, respectively; pH 7.6. Reaction was initiated by TK.

The observation that the mutation of TK at His103 leads to the increase of the one-substrate reaction rate and decrease of the two-substrate reaction rate, so that they become equal, may be considered as experimental substantiation of the earlier advanced hypothesis [1,2] about the intermediate stabilising function of His103. The acceleration of the DHEThDP cleavage and deceleration of its formation rate caused by the mutation makes the stage of intermediate formation the rate-limiting for both (the one- and two-substrate) types of catalysis, thus equalising their overall rates.

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