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Theoretical model of interactions between ligand-binding sites in a dimeric protein and its application for the analysis of thiamine diphosphate binding to yeast transketolase

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Abstract

The binding of thiamin diphosphate (ThDP) to yeast dimeric apotransketolase (apoTK) is accompanied by the appearance of a band in the absorption spectrum with maximum at 320 nm. The saturation function has been analyzed using a scheme that involves binding of ThDP to each subunit followed by the conformational transition of this subunit. It is assumed that the binding of ThDP to one subunit may affect the conformational transition of the other subunit. Rigorous mathematical expressions describing the dependence of the optical absorption on the total concentration of ThDP are first developed. Equilibrium constants and corresponding rate constants for the binding of ThDP to apoTK have been estimated. The negative cooperativity in the ThDP binding has been characterized by the function reflecting the dependence of the conformational change on the saturation of apoTK by ThDP.

Keywords: Transketolase; Negative cooperative interaction; Theoretical model; Thiamin diphosphate

1. Introduction

The elucidation of the mechanism of interactions between ligand-binding sites in oligomeric proteins remains one of the important problems in modern structural biochemistry. A number of models describing the interaction between ligand-binding sites in terms of the conformational changes in oligomeric protein subunits have been developed [1–6]. Among approaches commonly used to decipher the mechanisms of the allosteric interactions, are studies of the relations between the ligand concentration, on the one hand, and the protein saturation by the ligand or ligand-induced conformational changes in the protein molecule, on the other hand. However, most informative is the analysis of the correlation between the degree of the conformational changes and the degree of the protein saturation. This approach is used rather rarely. For example, Durchshlag and coauthors [7] analyzed the dependence of the volume decrease of the yeast glyceraldehyde-3-phosphate dehydrogenase molecule on the saturation degree of the enzyme by NAD. Another example is the work of Noel and Schumaker [8] who studied the change in the friction coefficient of glyceraldehyde-3-phosphate dehydrogenase from rabbit skeletal muscle as a function of the saturation degree of the enzyme by NAD.

A great number of investigations deal with the interactions between active sites in transketolase (TK) from *Saccharomyces cerevisiae* [9–13]. TK catalyzes a reversible transfer of a two-carbon fragment (glycol aldehyde residue) from ketose (substrate–donor) to aldose (substrate–acceptor). Thiamine diphosphate (ThDP) and divalent cations, such as Ca^{2+} and Mg^{2+}, act as cofactors [14,15]. TK from *S. cerevisiae* is a homodimer having two active sites with identical catalytic

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activities [16–18]. The active sites are characterized by negative cooperativity for coenzyme binding in the presence of Ca^{2+} [9,10,17,18]. In the presence of Mg^{2+}, the behavior of the active centers with respect to ThDP is either equivalent [19,20] or slightly different [11]. A donor substrate induces the appearance of cooperativity in the presence of Mg^{2+} (if the cooperativity was absent previously) and enhances the cooperativity observed in the presence of Ca^{2+} [13].

The interaction of ThDP with apoTK involves at least two stages [21]:

$$\mathrm{TK} + \mathrm{ThDP} \overset{K_d}{\longleftrightarrow} \mathrm{TK}\cdot\cdot\cdot\mathrm{ThDP} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftarrows}} \mathrm{TK}^* - \mathrm{ThDP}. \quad (1)$$

The first step proceeds fast and results in the formation of the catalytically inactive complex TK···ThDP. The slow conformational change of this complex produces the catalytically active holoenzyme TK*−ThDP. It is worthwhile to note that the formation of (TK*−ThDP) may be followed by the change in the absorbance at the wavelength range of 300–340 nm [11–13,22]; this change characterizes the conformational changes in the active sites of TK.

Kovina at al. [23] developed the kinetic scheme taking into account the interactions between the ThDP-binding sites in the TK dimeric molecule. The proposed scheme involves the stage of the attachment of the ThDP molecule to the binding site followed by a reversible isomerization of the whole complex. According to this scheme, the negative cooperativity in ThDP binding is due to the different rates of the reverse reactions for the conformational transitions of the two active sites.

Here we analyze the interactions between the ligand-binding sites in a dimeric protein molecule, plot the conformational changes versus protein saturation, and by means of these pots, identify types and strengths of the cooperative interactions between the ligand-binding sites. The results of the theoretical analysis are used for the interpretation of the experimental data on the ThDP–TK binding.

2. Theoretical analysis of the interactions between ligand-binding sites in transketolase

Consider a ligand binding to a dimeric protein. The ligand attachment to each binding site involves a stage of the complex formation followed by the isomerization stage. When applied to TK, this scheme acquires the form presented in Fig. 1 [24].

Two ThDP-binding sites in the transketolase molecule are originally equivalent and characterized by the dissociation constant K_d. The equilibrium constant for subsequent isomerization K_1 is independent of whether the ligand-binding site in the neighboring subunit is occupied or not. The states of subunits E in the LĒE and EĒL forms are characterized by the same affinity (K_d) to ThDP identical to that for the subunits in the initial EE form. However, it is assumed that the presence of conformationally changed subunit in the dimer affects the conformational transition of the E subunit (in other words, the isomerization constant K_2 is different from the isomerization constant K_1).

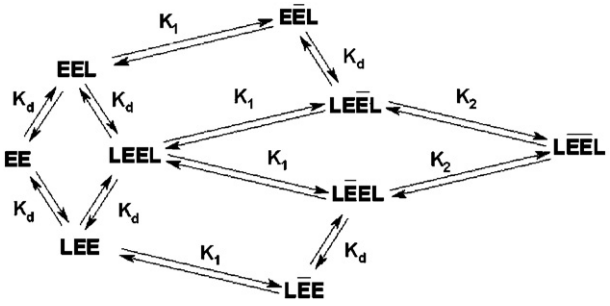

Fig. 1. The binding of ThDP to apoTK. EE is the initial catalytically inactive dimer of apoTK, L is ThDP, EĒ and ĒE are the dimeric enzyme forms containing one conformationally changed subunit Ē, ĒĒ is the dimeric enzyme form with two conformationally changed subunits.

For the scheme under discussion the equilibrium constants are expressed as follows:

$$K_d = \frac{[\mathrm{EE}][\mathrm{L}]}{[\mathrm{EEL}]} = \frac{[\mathrm{EEL}][\mathrm{L}]}{[\mathrm{LEEL}]} = \frac{[\mathrm{E\bar{E}L}][\mathrm{L}]}{[\mathrm{LE\bar{E}L}]}, \quad (2)$$

$$K_1 = \frac{[\mathrm{EEL}]}{[\mathrm{E\bar{E}L}]} = \frac{[\mathrm{LEEL}]}{[\mathrm{LE\bar{E}L}]}, \quad (3)$$

$$K_2 = \frac{[\mathrm{L\bar{E}EL}]}{[\mathrm{L\bar{E}\bar{E}L}]}, \quad (4)$$

where [L] is the concentration of free ThDP.

The concentrations of the ThDP-containing forms are related to the initial dimer concentration [EE] as follows:

$$[\mathrm{EEL}] = \frac{[\mathrm{EE}][\mathrm{L}]}{K_d}, \quad (5)$$

$$[\mathrm{LEEL}] = \frac{[\mathrm{EEL}][\mathrm{L}]}{K_d} = \frac{[\mathrm{EE}][\mathrm{L}]^2}{K_d^2}, \quad (6)$$

$$[\mathrm{E\bar{E}L}] = \frac{[\mathrm{EEL}]}{K_1} = \frac{[\mathrm{EE}][\mathrm{L}]}{K_1 K_d}, \quad (7)$$

$$[\mathrm{LE\bar{E}L}] = \frac{[\mathrm{E\bar{E}L}][\mathrm{L}]}{K_d} = \frac{[\mathrm{EE}][\mathrm{L}]^2}{K_1 K_d^2}, \quad (8)$$

$$[\mathrm{L\bar{E}\bar{E}L}] = \frac{[\mathrm{LE\bar{E}L}]}{K_2} = \frac{[\mathrm{EE}][\mathrm{L}]^2}{K_2 K_1 K_d^2}. \quad (9)$$

The type of interactions between the ThDP-binding sites is determined by the relationship between the constants K_1 and K_2. The case $K_2 > K_1$ corresponds to negative cooperative interactions between the ligand-binding sites. The case $K_2 < K_1$ corresponds to positive cooperative interactions.

The degree of saturation (Y) of the dimeric enzyme by the ligand is determined as the ratio of the sum of the ThDP-

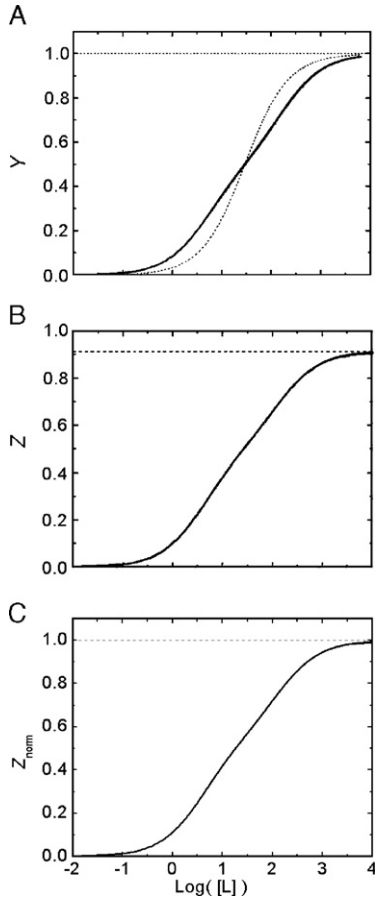

Fig. 2. Theoretical dependencies of the saturation degree Y (A), the degree of the conformational changes Z (B), and the normalized Z value ($Z_{norm}=Z/Z_{lim}$) (C) on the logarithm of the free ligand concentration [L] calculated from Eqs. (10), (11) and (13) at $K_d=1000$, $K_1=0.01$, and $K_2=0.1$ (the logarithmic scale is used for the abscissa axis). The dotted curve in panel A corresponds to the hyperbolic function (14) at $[L]_{0.5}=30.1$ μM. The dimension of [L] is μM.

containing enzyme forms to the total concentration of the dimer:

$$Y=\frac{[EEL]+[LEE]+[E\bar{E}L]+[L\bar{E}E]+[L\bar{E}EL]+[LE\bar{E}L]+[L\bar{E}\bar{E}L]}{2[E]_0}=\frac{\left(1+\frac{1}{K_1}\right)\frac{[L]}{K_d}+\left(1+\frac{2}{K_1}+\frac{1}{K_1K_2}\right)\frac{[L]^2}{K_1K_d^2}}{1+\left(1+\frac{1}{K_1}\right)\frac{2[L]}{K_d}+\left(1+\frac{2}{K_1}+\frac{1}{K_1K_2}\right)\frac{[L]^2}{K_d^2}}, \quad (10)$$

where $[E]_0$ is the total enzyme concentration calculated per subunit. The Y value varies from 0 to 1, depending on the free ligand concentration; the limiting value of Y is 1 at $[L]\to\infty$.

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

$$Z=\frac{[E\bar{E}L]+[L\bar{E}E]+[L\bar{E}EL]+[LE\bar{E}L]+[L\bar{E}\bar{E}L]}{2[E]_0}=\frac{\frac{[L]}{K_1K_d}+\left(1+\frac{1}{K_2}\right)\frac{[L]^2}{K_1K_d^2}}{1+\left(1+\frac{1}{K_1}\right)\frac{2[L]}{K_d}+\left(1+\frac{2}{K_1}+\frac{1}{K_1K_2}\right)\frac{[L]^2}{K_d^2}}. \quad (11)$$

At $[L]\to\infty$, Z approaches the limiting value:

$$Z_{lim}=\frac{1+K_2}{1+2K_2+K_1K_2}. \quad (12)$$

It is convenient to introduce a normalized Z value:

$$Z_{norm}=Z/Z_{lim}=\frac{(1+2K_2+K_1K_2)\left\{\frac{[L]}{K_1K_d}+\left(1+\frac{1}{K_2}\right)\frac{[L]^2}{K_1K_d^2}\right\}}{(1+K_2)\left\{1+\left(1+\frac{1}{K_1}\right)\frac{2[L]}{K_d}+\left(1+\frac{2}{K_1}+\frac{1}{K_1K_2}\right)\frac{[L]^2}{K_d^2}\right\}}. \quad (13)$$

Z_{norm} varies from 0 to 1 as the ligand concentration grows.

Consider for example the case of negative cooperativity ($K_2>K_1$). Fig. 2(A) and (B) show the dependencies of the saturation degree Y and the degree of the conformational change on the free ligand concentration [L], given that $K_d=1000$ μM, $K_1=0.01$, $K_2=0.1$. For the curve presented in Fig. 2(A) the value of [L], at which $Y=0.5$ ($[L]=[L]_{0.5}$), was found to be 30.1 μM. To demonstrate the deviation of the theoretical curve from the hyperbolic law, we have drawn a hyperbola:

$$Y_{hyp}=\frac{[L]/[L]_{0.5}}{1+[L]/[L]_{0.5}}. \quad (14)$$

As can be seen from Fig. 2(A), the theoretical curve corresponding to the case of negative cooperativity has a lesser slope in the inflexion point than the hyperbolic curve. As seen in Fig. 2(B), the limiting value of Z, which is reached at $[L]\to\infty$, is less than unity: $Z_{lim}=0.915$. Fig. 2(C) shows the dependence of the normalized Z value ($Z_{norm}=Z/Z_{lim}$) on the free ligand concentration.

The dependencies of Y and Z_{norm} on [L] can be presented as a 3D plot (Fig. 3). The XY projection corresponds to the dependence of Y on [L]; the XZ projection, to the dependence of Z_{norm} on [L]; and the YZ projection, to the dependence of Z_{norm} on Y.

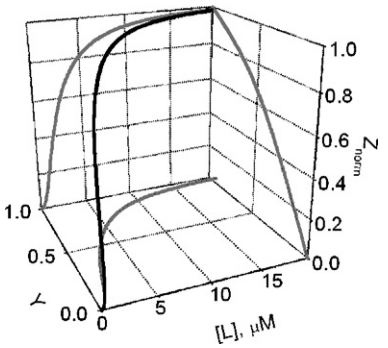

Fig. 3. The 3D plot demonstrating the dependencies of the saturation degree Y and the normalized degree of the conformational changes Z_{norm} on the free ligand concentration [L] ($K_d=800$, $K_1=0.01$, $K_2=1$).

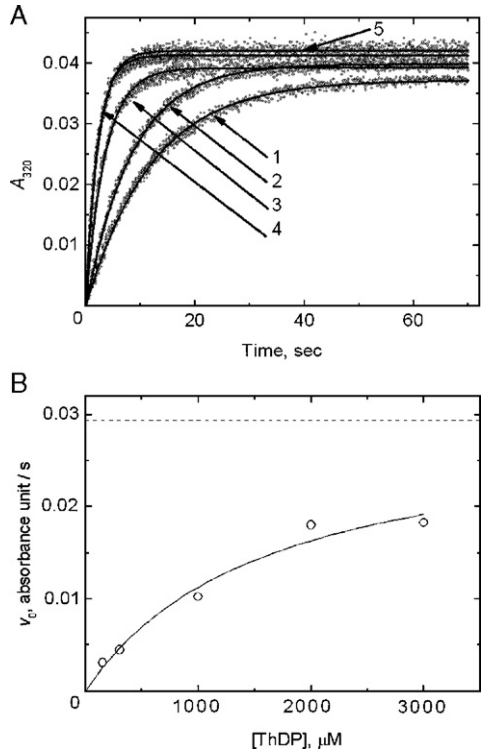

Fig. 4. The kinetics of the binding of ThDP to TK from *Saccharomyces cerevisiae* in the presence of 2.5 mM $MgCl_2$. The experiments were performed at the protein concentration of 6.75 μM (dimer) in 50 mM glycyl–glycine buffer (pH 7.6) at 25 °C. (A) The kinetic curves were obtained at the following ThDP concentrations: 150 (1), 300 (2), 1000 (3), 2000 (4), and 3000 μM (5) [25]. A_{320} is the optical absorbance at 320 nm. Solid curves are calculated from Eq. (28). (B) The dependence of the initial rate of the ThDP binding to TK (v_0) on the initial concentration of ThDP. Points are the experimental data, solid curve is calculated from Eq. (15). The horizontal dotted line corresponds to the limiting value of v_0 at [ThDP]→∞.

The degree of the conformational changes (Z) plotted vs. the degree of the protein saturation by the ligand (Y) is useful for the analysis of the cooperative interactions between the ligand binding sites in an allosteric protein. In Appendices A and B we analyze the shape of the curves Z_{norm} vs. Y, obtained for two well-known models of the allosteric enzymes, namely for the Monod–Wyman–Changeux and Koshland–Némethy–Filmer models.

3. Analysis of experimental data using the new theoretical model

We have used the theoretical considerations presented in the previous section for the analysis of the interactions between cofactor-binding sites in TK. To determine the dissociation constant K_d, we analyzed the kinetic curves of the ThDP binding obtained by Esakova et al. [25]. Fig. 4(A) shows the kinetics of the ThDP binding in the presence of 2.5 mM $MgCl_2$. Based on these kinetic data we calculated the dependence of the initial rate of the ThDP binding (v_0) on the ThDP initial concentration (Fig. 4(B)). This dependence is described by the hyperbolic function:

$$v_0 = \frac{v_{0,\mathrm{lim}}[\mathrm{ThDP}]}{K_d + [\mathrm{ThDP}]}, \tag{15}$$

where $v_{0,\mathrm{lim}}$ is the limiting value of v_0 at [ThDP]→∞ and K_d is the microscopic dissociation constant for the TK···ThDP complex (Eq. (1)). When analyzing the dependence of v_0 on [ThDP], we obtained the following values of $v_{0,\mathrm{lim}}$ and K_d: $v_{0,\mathrm{lim}}=0.0029\pm0.0004$ absorbance unit/s, and $K_d=1600\pm100$ μM.

The $v_{0,\mathrm{lim}}$ value characterizes the rate of the conformational transition EEL→EĒL (see scheme in Fig. 5):

$$v_{0,\mathrm{lim}} = 2\varepsilon k_{+1}[\mathrm{E}]_0, \tag{16}$$

where ε is the extinction coefficient for the complex of ThDP with the conformationally changed subunit, and k_{+}sub $_1$ is the rate constant for the conformation transition EEL→EĒL or LEE→LĒE.

To analyze the saturation of TK by ThDP (Eq. (10)) in the presence of 2.5 mM $MgCl_2$, we used the value of K_d obtained from the stopped-flow kinetic data. The saturation function obtained is shown in Fig. 6. To describe the dependence of A_{320} on the ThDP total concentration, we used Eqs. (10) and (11).

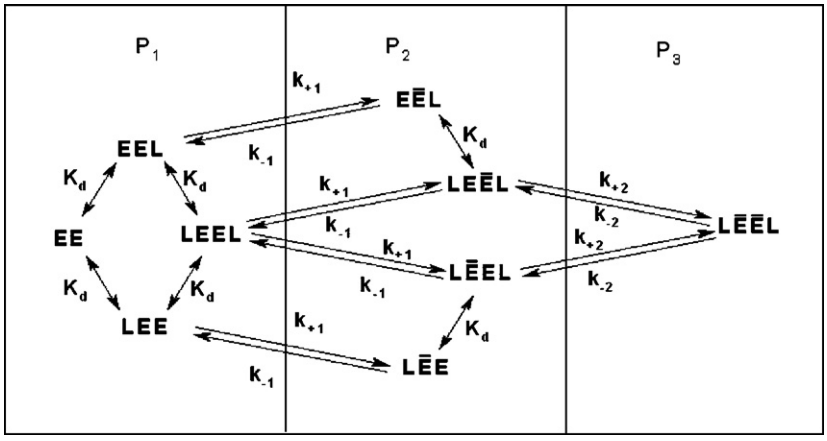

Fig. 5. The kinetic scheme of the binding of ThDP to apoTK. P_1, P_2, and P_3 are the pools of the enzyme-containing forms determined by Eqs. (19)–(21).

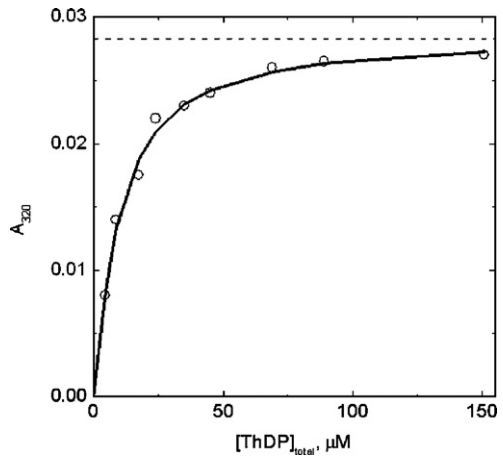

Fig. 6. Saturation of TK by ThDP in the presence of 2.5 mM $MgCl_2$ [13]. The experiments were performed at the protein concentration of 6.75 μM (dimer) in 50 mM glycyl–glycine buffer, pH 7.6, at 25 °C. A_{320} is absorbance at 320 nm. $[ThDP]_{total}$ is the total concentration of ThDP. Points are the experimental data. Solid curve is calculated from Eq. (18). The dotted horizontal line is the limiting value of A_{320} at $[ThDP] \to \infty$.

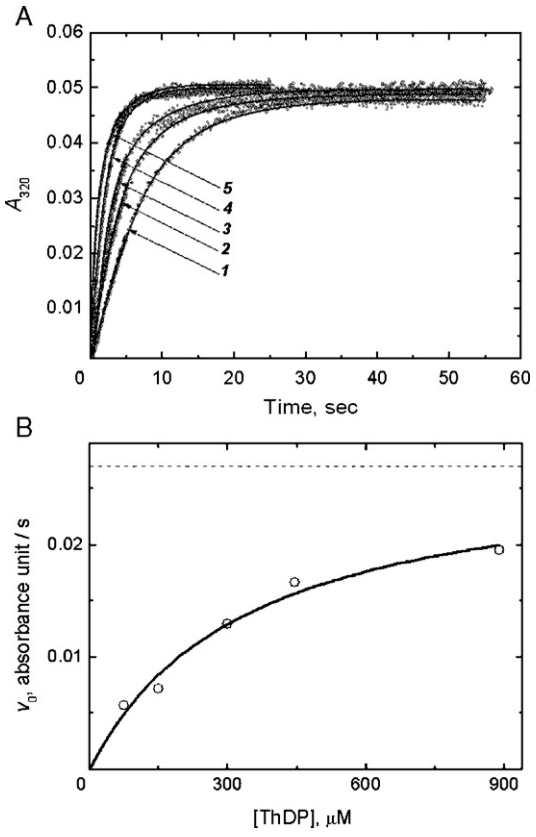

Fig. 7. The kinetics of the ThDP binding to TK from *Saccharomyces cerevisiae* in the presence of 2.5 mM $CaCl_2$. The experiments were performed at the protein concentration of 6.75 μM (dimer) in 50 mM glycyl–glycine buffer, pH 7.6, at 25 °C. (A) The kinetic curves were obtained at the following concentrations of ThDP: 75 (1), 150 (2), 300 (3), 445 (4) and 890 μM (5) [25]. A_{320} is optical absorbance at 320 nm. Solid curves are calculated from Eq. (28). (B) The dependence of the initial rate of the ThDP binding to TK (v_0) on the initial concentration of ThDP. Points are the experimental data, solid curve is calculated from Eq. (15). The horizontal dotted line corresponds to the limiting value of v_0 at $[ThDP] \to \infty$.

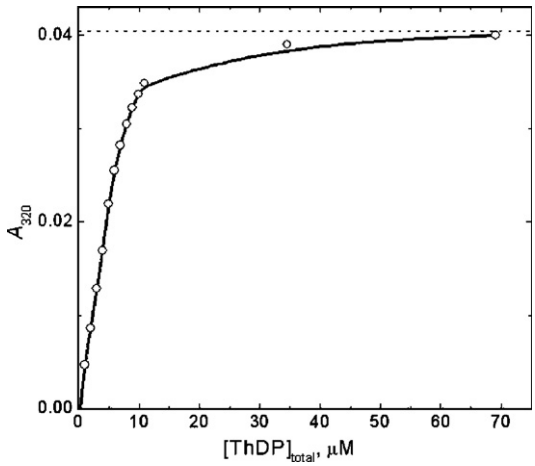

Fig. 8. Spectrophotometric titration of TK by ThDP in the presence of 2.5 mM $CaCl_2$ [26]. The experiments were performed at the protein concentration of 6.75 μM (dimer) in 50 mM glycyl–glycine buffer, pH 7.6, at 25 °C. A_{320} is optical absorbance at 320 nm. Points are the experimental data. The solid curve is calculated from Eq. (18).

The expression for Z contains the concentration of free ligand [L] which is related with $[L]_{total}$ as follows:

$$[L] = [L]_{total} - 2\varepsilon[E]_0 Y. \tag{17}$$

The increase in the optical absorbance (A) in the course of titration of TK by ThDP can be calculated this way:

$$A = 2\varepsilon[E]_0 Z. \tag{18}$$

To fit the theoretical Eqs.), (17) and (18 to the experimental data, we used the program Scientist (MicroMath, USA). The following values were obtained: $K_1 = 0.0006 \pm 0.0002$, $K_2 = 0.0066 \pm 0.0007$, $\varepsilon = 0.00269 \pm 0.00002$ μM^{-1} cm^{-1}.

Using these parameters, we further analyzed the kinetic data on the ThDP–TK binding presented in Fig. 4(A). First of all, we calculated the value of k_{+1} using Eq. (16): $k_{+1} = v_{0,lim}/2\varepsilon[E]_0 = 1.33 \pm 0.2$ s^{-1}. The rate constant k_{-1} can be calculated as $k_{-1} = k_{+1}K_1$, which gives the k_{-1} value of 0.0008 ± 0.0001 s^{-1}. To calculate the rate constants k_{+2} and k_{-2}, we derived kinetic equations according to the scheme presented in Fig. 5. First of all, we separated all the enzyme-containing forms into three pools:

$$P_1 = [EE] + [LEE] + [EEL] + [LEE] \tag{19}$$

$$P_2 = [E\bar{E}L] + [L\bar{E}E] + [LE\bar{E}L] + [L\bar{E}EL] \tag{20}$$

$$P_3 = [L\bar{E}\bar{E}L]. \tag{21}$$

The sum of all pools is equal to $[E]_0$. Conformational changes of the enzyme upon its transitions between pools are described by the system of differential equations:

$$\begin{cases} \frac{dP_1}{dt} = \gamma_{[E\bar{E}L]}k_{-1}P_2 + \gamma_{[L\bar{E}\bar{E}L]}k_{-1}P_2 - \gamma_{[EEL]}k_{+1}P_1 - 2\gamma_{[LEEL]}k_{+1}P_1 \\ \frac{dP_2}{dt} = -\gamma_{[E\bar{E}L]}k_{-1}P_2 - \gamma_{[L\bar{E}\bar{E}L]}k_{-1}P_2 + \gamma_{[EEL]}k_{+1}P_1 + 2\gamma_{[LEEL]}k_{+1}P_1 - \gamma_{[L\bar{E}\bar{E}L]} + k_{+2}P_2 + 2k_{-2}P_3 \\ \frac{dP_3}{dt} = \gamma_{[L\bar{E}\bar{E}L]}k_{+2}P_2 - 2k_{-2}P_3 \end{cases} \tag{22}$$

Table 1
Equilibrium and kinetic constants for the ThDP binding to TK in the presence of Mg^{2+} and Ca^{2+} in 50 mM glycyl–glycine buffer, pH 7.6, at 25 °C

Cations	K_d (μM)	K_1	k_{+1} (s^{-1})	k_{-1} (s^{-1})	K_2	k_{+2} (s^{-1})	k_{-2} (s^{-1})	ε (μM^{-1} cm^{-1})
Mg^{2+}	1600±100	0.0006±0.0002	1.33±0.2	0.0008±0.0001	0.0066±0.0007	0.42±0.01	0.0028±0.0001	0.00269±0.00002
Ca^{2+}	340±80	–	0.8±0.1	–	0.0013±0.0001	0.23±0.05	0.00030±0.00004	0.00375±0.00002

In this equations $\gamma_{[EEL]}$, $\gamma_{[LEEL]}$, $\gamma_{[E\bar{E}L]}$, $\gamma_{[L\bar{E}L]}$ are the portions of the corresponding enzyme-containing forms in the pools:

$$\gamma_{[EEL]} = \frac{2K_d[L]}{(K_d^2 + 2K_d[L] + [L]^2)} \quad (23)$$

$$\gamma_{[LEEL]} = \frac{[L]^2}{(K_d^2 + 2K_d[L] + [L]^2)} \quad (24)$$

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

$$\gamma_{[LE\bar{E}L]} = \frac{[L]}{(K_d + [L])}. \quad (26)$$

Taking into account the expression for the dissociation constant K_d

$$K_d = \frac{2\gamma_{[EE]}[L]}{\gamma_{[EEL]}} = \frac{\gamma_{[EEL]}[L]}{2\gamma_{[LEEL]}} = \frac{\gamma_{[E\bar{E}L]}[L]}{\gamma_{[LE\bar{E}L]}} \quad (27)$$

and the equations of material balance $(\gamma_{[EE]}+\gamma_{[EEL]}+\gamma_{[LEEL]})=1$ and $(\gamma_{[E\bar{E}L]}+\gamma_{[LE\bar{E}L]})=1$, we can obtain the following expression for the time-course of the optical absorbance change:

$$\frac{dA}{dt} = \varepsilon\left(\frac{dP_2}{dt} + 2\frac{dP_3}{dt}\right). \quad (28)$$

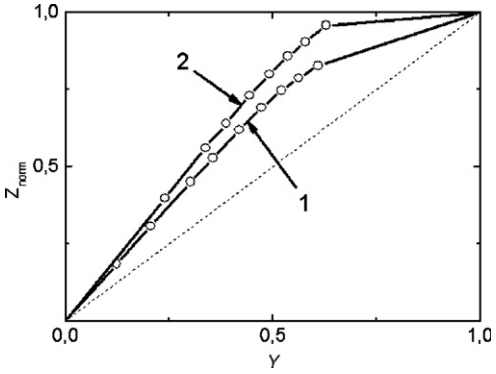

Fig. 9. Plots Z_{norm} vs. Y for the ThDP binding to TK in the presence 2.5 mM $MgCl_2$ (curve 1) and 2.5 mM $CaCl_2$ (curve 2). Curves 1 and 2 were calculated from Eqs. (11) and (12) using the values of K_d, K_1, and K_2 given in Table 1.

This equation was used for the analysis of the kinetic curves of the ThDP–TK binding in the presence of 2.5 mM $MgCl_2$. We took into account that $k_{-2}=k_{+2}K_2$. The rate constant k_{+2} was calculated for each kinetic curve. Fitting was performed using the program Scientist. The average value of k_{+2} was found to be 0.42±0.01 s^{-1}. Knowing K_2, we calculated the rate constant k_{-2}: $k_{-2}=0.0028\pm0.0001$ s^{-1}.

By analogy, we analyzed the data on the ThDP–TK binding in the presence of 2.5 mM $CaCl_2$. Fig. 7(A) shows the stopped-flow data on the binding kinetics. Based on these data we constructed the dependence of the initial rate of the ThDP binding on the ThDP concentration. The analysis of this dependence using Eq. (15) gave $K_d=340\pm80$ μM and $v_{0,lim}=0.027\pm0.002$ absorbance unit/s. When analyzing the saturation function of TK by ThDP (Fig. 8), we assumed that K_1 is very small (10^{-8}). Eq. (18) describing the dependence of A_{320} on $[ThDP]_{total}$ gave $\varepsilon=0.00375\pm0.00002$ μM^{-1} cm^{-1} and $K_2=0.0013\pm0.0001$. It should be noted that at $K_1<10^{-8}$, ε and K_2 do not change. Knowing ε, we can calculate the rate constant k_{+1} using Eq. (16): $k_{+1}=0.8\pm0.1$ s^{-1}. When fitting the theoretical Eq. (28) to the experimental data, we obtained the following values of the rate constants k_{+2} and k_{-2}: $k_{+2}=0.23\pm0.05$ s^{-1} and $k_{-2}=0.00030\pm0.00001$ s^{-1}. The parameters for the ThDP–TK binding in the presence 2.5 mM $MgCl_2$ and 2.5 mM $CaCl_2$ are summarized in Table 1.

Using the obtained values of K_d, K_1, and K_2, we plotted Z_{norm} vs. Y for the ThDP–TK binding in the presence of Mg^{2+} and Ca^{2+} (Fig. 9). The curves lie above the straight line passing at angle of 45°, which points to the negative cooperativity of the interactions between the ThDP binding sites in the dimeric TK molecule.

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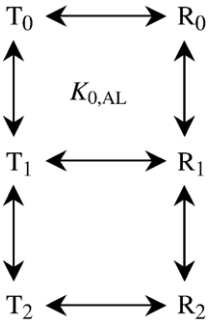
Appendix A. Analysis of the interactions between ligand-binding sites in the Monod–Wyman–Changeux model

In the Monod–Wyman–Changeux model [1], the interactions between the ligand-binding sites are mediated by the T↔R transition of the oligomeric protein (T and R is the tense and relaxed state, respectively). It is assumed that changes in the conformation of the subunits occur in a concerted manner.

The binding of the ligand to the dimeric protein is expressed by following scheme:



(A1)

Scheme 1. The binding of a ligand to a dimeric protein. T_0 and R_0 are the initial states of the protein. T_1 and R_1 are the complexes containing one ligand molecule. T_2 and R_2 are the complexes containing two ligand molecules.

In this scheme $K_{0,AL}$ is the allosteric constant: $K_{0,AL}=[T]_0/[R]_0$. K_T and K_R are the microscopic dissociation constants for the processes of the binding of the ligand to the T- and R-conformation, respectively. The degree of the protein saturation by the ligand depends on the ligand concentration the following way:

$$Y = \frac{[R_1] + 2[R_2] + [T_1] + 2[T_2]}{2([R_0] + [R_1] + [R_2] + [T_0] + [T_1] + [T_2])} = \frac{\frac{[L]}{K_R}\left(1 + \frac{[L]}{K_R}\right) + K_{0,AL}\frac{[L]}{K_T}\left(1 + \frac{[L]}{K_T}\right)}{\left(1 + \frac{[L]}{K_R}\right)^2 + K_{0,AL}\left(1 + \frac{[L]}{K_T}\right)^2}. \quad (A2)$$

The portion of the R form is expressed as:

$$\gamma_R = \frac{[R_0] + [R_1] + [R_2]}{[R_0] + [R_1] + [R_2] + [T_0] + [T_1] + [T_2]} = \frac{\left(1 + \frac{[L]}{K_R}\right)^2}{\left(1 + \frac{[L]}{K_R}\right)^2 + K_{0,AL}\left(1 + \frac{[L]}{K_T}\right)^2}. \quad (A3)$$

At [L]=0, the γ_R value approaches the limiting value:

$$\gamma_{R,0} = \frac{1}{1 + K_{0,AL}}. \quad (A4)$$

At [L]$\rightarrow\infty$, the γ_R value approaches the limiting value:

$$\gamma_{R,lim} = \frac{1}{1 + K_{0,AL}\left(\frac{K_R}{K_T}\right)^2}. \quad (A5)$$

The ratio of $(\gamma_R - \gamma_{R,0})$ to $(\gamma_{R,lim} - \gamma_{R,0})$ can be considered as a degree of the conformational changes in subunits:

$$Z_{norm} = \frac{(\gamma_R - \gamma_{R,0})}{(\gamma_{R,lim} - \gamma_{R,0})}. \quad (A6)$$

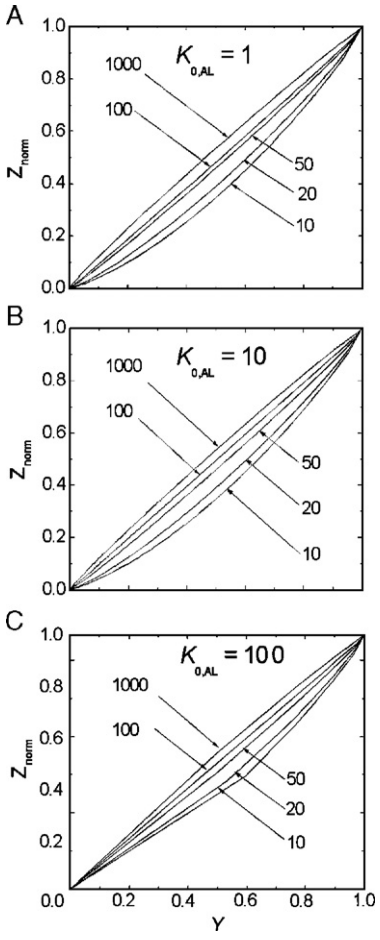


Fig. 10. Theoretical plots Z_{norm} vs. Y for the dimeric protein according to the Monod–Wyman–Changeux model. The dependencies of Z_{norm} on Y are calculated at $K_{0,AL}$ equal to 1 (A), 10 (B), and 100 (C). Numbers near the curves correspond to the K_R/K_T values.

Fig. 10 shows the theoretical dependencies of the Z_{norm} value on the degree of the saturation of the protein by the ligand calculated at $K_{0,AL}$ equal to 1, 10, and 100 and at various values of the K_R/K_T ratio. As can be seen from the plots Z_{norm} vs. Y, the interactions between the ligand binding sites clearly exhibit positive cooperativity, since the values of the K_R/K_T ratio are relatively low and the curves lie below the straight line passing at an angle of 45°. Only this type of deviations from the linear $Z_{norm} - Y$ relation, is possible in the Monod–Wyman–Changeux model.

Appendix B. Analysis of the interactions between ligand-binding sites for the Koshland–Némethy–Filmer model

According to the Koshland–Némethy–Filmer model, the binding of a ligand to the subunit in the protein oligomer is accompanied by the change in the subunit conformation. The conformational change of one subunit may affect the stability of the conformations of the neighboring subunits with a sequential change in their affinity for the ligand. It is assumed that each subunit can occur in one of two possible conformations (A or

B). Let K_t be the equilibrium constant for the conformational change A ↔ B:

$$K_t = \frac{[A]}{[B]}. \quad (B1)$$

We further designate the microscopic dissociation constant or the complex of the ligand with an individual subunit in the B conformation as K_L:

$$K_L = \frac{[B][L]}{[BL]}. \quad (B2)$$

Binding of the ligand by the subunit is thus described by two equilibrium constants s(K_t and K_L):

$$K_t K_L = \frac{[A][L]}{[BL]}. \quad (B3)$$

In order to make allowance for the interactions between subunits, constants K_{AA}, K_{AB}, K_{BB} are introduced. K_{AA} is the interaction constant between the subunits, which do not contain bound ligand, and is assumed to be equal to unity. K_{AB} is a measure of the strength of the AB interaction relative to the AA interaction:

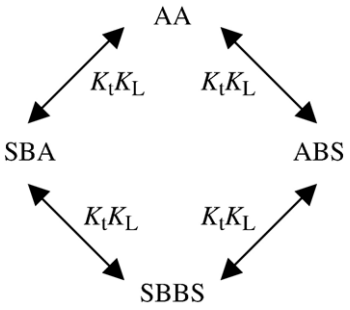
$$K_{AB} = \frac{[AB][A]}{[AA][B]}, \quad (B4)$$

where AB and AA are the interacting subunits, and A and B are the non-interacting subunits.

The constant K_{BB} is used to describe the interaction between two subunits in the B conformation:

$$K_{AB} = \frac{[BB][A][A]}{[AA][B][B]}. \quad (B5)$$

Consider the binding of the ligand by the dimeric protein:



(B6)

The dependence of the degree of the protein saturation by the ligand on the free ligand concentration has the following form (see [4]):

$$Y = \frac{[ABL] + [LBA] + 2[LBBL]}{[AA] + [ABL] + [LBA] + [LBBL]} = \frac{2K_{AB}\frac{[L]}{K_sK_t} + K_{BB}\left(\frac{[L]}{K_sK_t}\right)^2}{1 + K_{AB}\frac{[L]}{K_sK_t} + K_{BB}\left(\frac{[L]}{K_sK_t}\right)^2}. \quad (B7)$$

Inspection of this equation shows that when $K_{BB} > 2K_{BB}^2$, the plots Y vs. [L] become sigmoid. This case corresponds to the positive cooperative interactions between the ligand-binding sites. When $K_{BB} > 2K_{BB}^2$, the dependencies of Y on [L] are convex in the reciprocal coordinates (negative cooperativity).

Since the binding of a ligand to the subunit in the protein oligomer for the Koshland–Némethy–Filmer model is accompanied by the change in the conformation of the subunit, the dependence of the degree of the conformational change of the protein Z (i.e., the portion of the B conformation) on the free ligand concentration is identical to the degree of the protein saturation by the ligand. Thus, for the Koshland–Némethy–Filmer model the plot Z vs. Y is a straight line with the slope equal unity.

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