Kinetic mechanism of active site non-equivalence in transketolase

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Received 6 October 1997

Abstract The two-step mechanism of coenzyme (TDP) binding to apotransketolase has been examined by kinetic modeling, and the rate and equilibrium constants for each binding step for two active sites have been determined. The dissociation constants for the primary fast binding step and the forward rate constants for the secondary slow binding step have been shown to be similar for two active sites. The backward rate constants for the secondary binding step are different for two active sites, providing the kinetic mechanism of their non-equivalence in TDP binding.

Key words: Transketolase; Thiamine diphosphate; Kinetic modeling

1. Introduction

The dimeric molecule of transketolase (TK) contains two binding sites for the cofactor thiamine diphosphate (TDP), whose binding at each of them involves at least two steps [1]. TDP binding in the presence of Ca^{2+} is characterized by negative cooperativity; the dissociation constants for TDP binding to two sites differ about 10-fold [2–5]. In the present work, we apply kinetic modeling to the analysis of apoTK interaction with TDP to evaluate the individual kinetic parameters characterizing the two steps of TK and TDP interaction in order to determine the reason for active site non-equivalence in TDP binding.

2. Materials and methods

2.1. Materials

Baker's yeast TK with a specific activity of 17 U/mg was isolated as previously described [6]. The enzyme was homogeneous according to SDS-PAGE. TK concentration was determined spectrophotometrically, using $A^{1\%}_{1cm}$ of 14.5 at 280 nm [7]. TDP was purchased from Serva (Germany), $CaCl_2$ from Sigma (USA).

2.2. Kinetic measurements

TDP binding to apoTK results in an absorption maximum at 315–320 nm [8–11], whose intensity is proportional to the amount of the reconstituted holoenzyme [12–14]. The kinetics of the absorbance increase was recorded in an Aminco DW-2000 spectrophotometer operated in a two-wavelength mode (the reference wavelength was 360 nm) until the equilibrium level was attained (usually 10 min). The reconstitution was carried out in 10 mM Tris-HCl buffer, pH 7.35, containing 2 mM $CaCl_2$.

2.3. Kinetic model and calculations

TDP binding proceeds through two steps [1]. Taking into account that a molecule of TK is a homodimer with two identical active sites [15], and, hence, interacts with two TDP molecules, the overall binding reaction can be described by Scheme 1, where E is enzyme monomer, T is TDP, ET is the primary complex, ET* is the active holoenzyme (the only species with the characteristic absorption maximum at 315 nm [3,14]), x_0–x_5 are the enzyme species with different active site status and their concentrations, K^1_d and K^2_d are the dissociation constants for the primary apoTK complexes with one and two TDP molecules, respectively, k_i and k_{-i} ($i = 1,2,4$) are the rate constants for the corresponding forward and reverse reactions of the secondary TDP binding to the x_i species. Basing on X-ray crystallographic data [16], we assume that both apoTK sites are initially equivalent, hence the first TDP molecule is bound to any site with the same probability. The primary binding, which proceeds much faster than the secondary binding [1], is assumed to be a rapid equilibrium one. $K^3_d = K^3_d k_1 k_{-2}/k_{-1}k_1$ is the dissociation constant for x_4.

Scheme 1 is described by the following set of algebraic and ordinary differential equations:

$$2x_0[T]/x_1 = K^1_d \quad (1)$$

$$x_1[T]/2x_2 = K^2_d \quad (2)$$

$$x_3[T]/x_4 = K^3_d \quad (3)$$

$$dx_3/dt = k_1x_1 - k_{-1}x_3 \quad (4)$$

$$dx_4/dt = 2k_2x_2 - (k_{-2} + k_3)x_4 + k_{-3}x_5 \quad (5)$$

$$x_0 + x_1 + x_2 + x_3 + x_4 + x_5 = [E_0] \quad (6)$$

$$x_1 + x_3 + 2(x_2 + x_4 + x_5) + [T] = [T_0] \quad (7)$$

Integration of the differential equations was performed with a computer program (Selivanov, V.A., in preparation) using the Calahan method [17]. At each integration step, quasiequilibrium concentrations were recalculated with Eqs. 1–3, 6 and 7. Parameters K^1_d, K^2_d, k_i, k_{-1} were evaluated by fitting calculated curves to the experimental data as described below. The equilibrium fractions of the enzyme species and free and bound TDP concentrations were computed by setting the derivatives in Eqs. 4 and 5 to zero. The resulting algebraic equations were solved by the Newton method [18].

3. Results

The dependence of equilibrium holoenzyme concentration on total TDP concentration (Fig. 1) yielded an apparent dissociation constant of 0.6 μM for the second active site in dimeric TK molecule, a value similar to previous estimates [2,5].

To determine all eight parameters in Scheme 1 (K^1_d, K^2_d, k_1, k_{-1}, k_2, k_{-2}, k_3 and k_{-3}) one needs not only the equilibrium data presented in Fig. 1, but also the time dependence of the holoTK reconstitution at various TDP concentrations and initial holoTK percentage. The calculation procedure is illustrated below for model 1 (Table 1). In this model, we assumed that the primary TDP binding to one active site does not induce any conformational change in the other active site of TK, so that all the kinetic parameters for it remain unchanged. Therefore, $K^1_d = K^2_d$, $k_1 = k_2$, $k_{-1} = k_{-2}$. Only the secondary TDP binding to one site weakens TDP binding to the other site.

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Abbreviations: TK, transketolase; TDP, thiamine diphosphate

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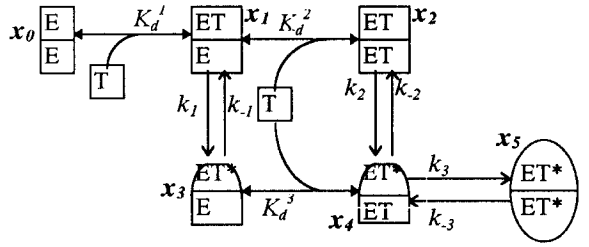

Scheme 1.

At step 1 of the calculation procedure, we evaluated the dissociation constants for the primary binding, by analysis of the initial reconstitution rates at two different TDP concentrations. According to Scheme 1, only three enzyme species (x_0, x_1, x_2) are present following TDP addition, since formation of other species requires the slow secondary step. Therefore, the secondary binding rate at zero time is proportional to the concentration of only x_1 and x_2 (to the term $k_1x_1+2k_2x_2$). The equilibrium between x_0, x_1 and x_2 at zero time is described by Eqs. 8–11:

$$2x_0[T]/x_1 = K_d^1 \quad (8)$$

$$x_1[T]/2x_2 = K_d^1 \quad (9)$$

$$x_0 + x_1 + x_2 = [E_0] \quad (10)$$

$$x_1 + 2x_2 + [T] = [T_0] \quad (11)$$

The system contains a single unknown parameter, $K_d^1 = K_d^2$, which could be determined from the initial reconstitution rates measured at two different initial TDP concentrations (Fig. 2). The ratio of the observed initial rates is equal to the ratio of the corresponding expressions $k_1x_1+2k_2x_2$ obtained by solving Eqs. 8–11. The value of K_d^1 obtained in this way was 100 μM.

At the second calculation step, we used $K_d^1 = K_d^2 = 100$ μM to determine the secondary binding equilibrium constants ($K^1 = k_1/k_{-1}$, $K^2 = k_2/k_{-2}$ and $K^3 = k_3/k_{-3}$), by fitting the dependence of equilibrium holoenzyme content on total TDP concentration (Fig. 1). The initial part of this dependence is determined by only one parameter, $K^1 = K^2$, since at low TDP concentration it is bound only to the first site. Increasing the $K^1 = K^2$ value to 1000 (curve 2) allowed fitting the first part of the experimental curve (the above value is only a lower limit, as further increasing $K^1 = K^2$ did not affect fit quality). The remaining part of the curve depends only on K^3, since the first site is already occupied. The best-fit value of K^3 was found to be about 100 (curve 3).

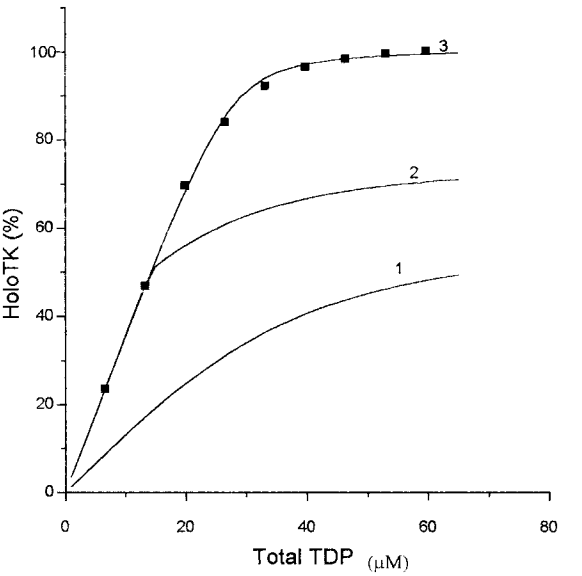

Fig. 1. Equilibrium formation of holoTK as a function of total TDP concentration. The squares are experimental points, the lines are calculated for $K_d^1 = K_d^2 = 100$ μM, $K^1 = k_1/k_{-1} = 1$, $K^2 = k_2/k_{-2} = 1$, $K^3 = k_3/k_{-3} = 1$, except for $K^1 = K^2 = 1000$ for curves 2, 3 and $K^3 = 100$ for curve 3. ApoTK concentration (monomer) = 28 μM.

At the third calculation step, values of $K_d^1 = K_d^2$, $K^1 = K^2$ and K^3 were used to estimate the rate constants for the secondary binding, by fitting the progress curves of holoTK reconstitution (Fig. 3). The first added portions of TDP were bound entirely to the first site, hence the response depended only on $k_1 = k_2$ (and $k_{-1} = k_1/K^1$), which could be evaluated from curve 1 to be 0.35 s^{-1} ($k_{-1} = k_{-2} = k_1/K^1 = 0.00035$ s^{-1}). Response to the second TDP addition is also determined by k_1, whereas the following additions to the mixture containing more than 50% of holoTK were bound solely by the second site and the corresponding kinetics is determined only by k_3 (and $k_{-3} = k_3/K^3$). The best-fit values obtained from curves 3–8 were as follows: $k_3 = 0.3$ s^{-1}, $k_{-3} = k_3/K^2 = 0.003$ s^{-1}.

Final verification of the evaluated parameters was done by examining the fit to the reconstitution kinetics measured in largely different conditions, in particular, at excess TDP, when both sites bind it simultaneously. Fig. 2 makes clear that the fit was satisfactory both at low (20 μM) and high (90 μM) total TDP concentrations.

In alternative models (Table 1), even primary TDP binding affects the second active site, decreasing its affinity to TDP. One should consider two possibilities: (a) $K_d^1 < K_d^2$, $K^1 = K^2$ (primary binding to the second site is affected) and (b) secondary binding to the second site is affected. In both cases, we assume that the analogous constants for the two sites differ at

Table 1
Kinetic models of TDK binding to TK

Model	Assumptions	Calculated parameter values							
		K_d^1 (μM)	K_d^2 (μM)	k_1 (s^{-1})	k_{-1} (s^{-1})	k_2 (s^{-1})	k_{-2} (s^{-1})	k_3 (s^{-1})	k_{-3} (s^{-1})
1	$K_d^1 = K_d^2$, $k_1 = k_2$, $k_{-1} = k_{-2}$	100	100	0.35	3.5×10^{-4}	0.35	3.5×10^{-4}	0.3	0.003
2	$10K_d^1 = K_d^2$, $k_1 = k_2$, $k_{-1} = k_{-2}$	250	2500	0.7	1.6×10^{-4}	0.7	1.6×10^{-4}	8	0.0048
3	$K_d^1 = K_d^2$, $k_1 = 10k_2$, $k_{-1} = k_{-2}$	250	250	0.7	2.3×10^{-4}	0.07	2.3×10^{-4}	8	0.004
4	$K_d^1 = K_d^2$, $k_1 = 10k_2$, $k_{-1} = 10k_{-2}$	250	250	0.7	2.3×10^{-4}	0.07	2.3×10^{-5}	0.8	0.004
5	$K_d^1 = K_d^2$, $k_1 = k_2$, $k_{-1} = 0.1k_{-2}$	250	250	0.3	10^{-6}	0.3	10^{-5}	0.8	0.004

least by a factor of 10 and check whether it is possible to find the parameter values which allow one to describe all experimental curves. When analyzing case (a), we assumed 10 $K_d^1 = K_d^2$ and used, as described above, the initial reconstitution rates measured at two different total TDP concentrations to determine K_d^1 to be 250 mM. Fitting all other curves yielded a set of other parameter values unique for the above K_d^1.

When analyzing case (b), one should consider three different effects of primary TDK binding to the first site on the secondary TDP binding to the second site: (1) only the forward rate constant (k_2) is decreased; (2) the forward and reverse rate constants (k_2 and k_{-2}) are decreased proportionally; (3) only the reverse rate constant is increased. For all of these cases, it was possible to find the best-fit set of parameters describing the experimental data (Figs. 1–3).

3.1. Comparison of different models

The five models of TDK binding tested in the present work are listed in Table 1. Model 1, described above, and models 3–5 assume no interaction between sites at the level of the primary binding ($K_d^1 = K_d^2$), whereas model 2 assumes such an interaction ($K_d^1 < K_d^2$). Besides, the rate constants for the secondary binding step in the first active site are different in models 3–5. By comparing the calculated parameter values, one can see that the backward rate constant for the second site (k_{-3}) is greater than the respective rate constants for the first site (k_{-1} and k_{-2}) for all the models tested. Simulated TDP binding plots (not shown) obtained by setting $k_{-3} = k_{-1} = k_{-2}$ revealed no binding cooperativity, confirming that it is a consequence of differences in these parameters.

4. Discussion

The results presented above suggest that the observed cooperativity in TDP binding to TK is associated with the backward reaction of secondary TDP binding, which indicates conformational instability of one of the active sites in holodimer. The most likely binding model (model 1) assumes that the two active sites present in enzyme dimer behave identically unless TDP binding to both sites is completed. In the final complex [$(ET^*)_2$ in Scheme 1], destabilization may take place with the same probability in any active site. We believe that the resulting asymmetric state with differently strained subunits is unstable and the enzyme molecule oscillates between two states in which different subunits are destabilized. Such an oscillation would explain the identical activity of the two active sites in holodimer [4].

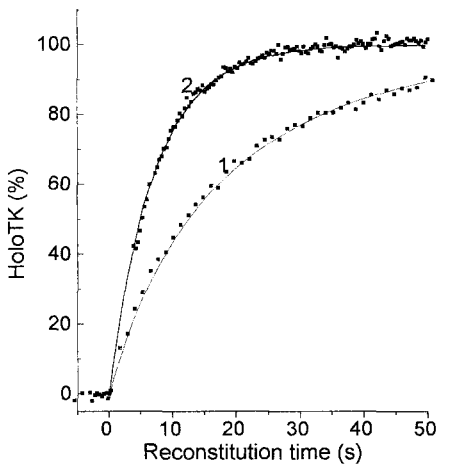

Fig. 2. Kinetics of holoTK reconstitution from 8 μM apoTK in response to a single large TDP addition. The squares are experimental points measured at 20 μM (lower curve) or 90 μM (upper curve) total TDP. The lines are calculated for $K_d^1 = K_d^2 = 100$ μM, $k_1 = k_2 = 0.35$ s^{-1}, $k_{-1} = k_{-2} = 0.00035$ s^{-1}, $k_3 = 0.3$ s^{-1}, $k_{-3} = 0.003$ s^{-1}.

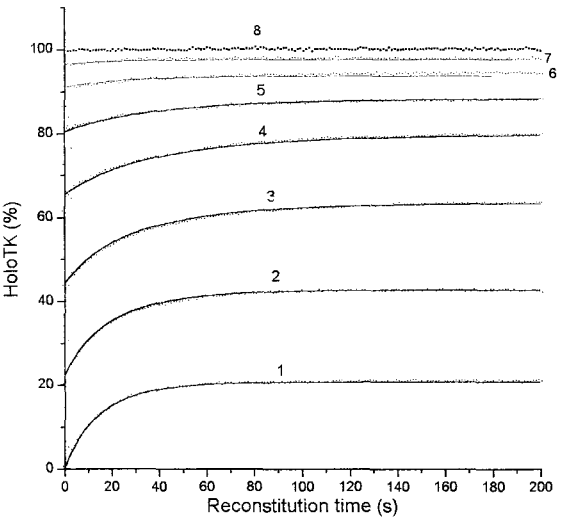

Fig. 3. Kinetics of holoTK reconstitution from 28 μM apoTK in response to multiple 6.6 μM TDP additions. The lines are calculated for $K_d^1 = K_d^2 = 100$ μM, $k_1 = k_2 = 0.35$ s^{-1}, $k_{-1} = k_{-2} = 0.00035$ s^{-1}, $k_3 = 0.3$ s^{-1}, $k_{-3} = 0.003$ s^{-1}. The measured values agreed within 5% with the calculated ones.

The facilitation of the backward transition could thus be explained by the strain in the enzyme upon binding two TDP molecules. Although no clear geometric difference between the active sites is seen in TK crystals, the distribution of the temperature factor is quite different in the two subunits and the mean standard deviation of residue position is significantly greater in the 'hot' subunit [15], supporting the 'strain' hypothesis. It is likely that the conformational difference between subunits is greater in solution versus crystals and the subunits have more freedom to interchange their states. Such a transition might involve two loops (residues 383–394 and 187–198) that have maximal temperature factor, maximal motility and asymmetry in the dimer molecule [15,19].

The hypothesis of permanent oscillations can explain the paradoxical discrepancy between slow TDP dissociation (some tens of minutes) [5] and rapid TDP exchange with medium (< 20 s) [20]. One can speculate that some of the TDP binding residues of one of the sites ('open') do not contact the bound TDP and can establish interactions with a new TDP molecule, which replaces the former one during site 'closing'. This replacement is the basis for TDP exchange with the medium and can occur at a rate comparable with the rate of the 'close-open' oscillations.

Acknowledgements: We are deeply grateful to Prof. A.A. Baykov for valuable critics and correction. This work was supported by the Russian Foundation for Basic Research (Grant 96-04-50730).

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