Effects of Free Ca^{2+} on Kinetic Characteristics of Holotransketolase

**Olga N. Solovjeva · Irina A. Sevostyanova ·
Vladimir A. Yurshev · Vitalii A. Selivanov ·
German A. Kochetov**

Abstract Catalytic activity has been demonstrated for holotransketolase in the absence of free bivalent cations in the medium. The two active centers of the enzyme are equivalent in both the catalytic activity and the affinity for the substrates. In the presence of free Ca^{2+} (added to the medium from an external source), this equivalence is lost: negative cooperativity is induced on binding of either xylulose 5-phosphate (donor substrate) or ribose 5-phosphate (acceptor substrate), whereupon the catalytic conversion of the bound substrates causes the interaction between the centers to become positively cooperative. Moreover, the enzyme total activity increase is observed.

Keywords Calcium ions · Kinetic parameters · Negative cooperativity · Positively cooperative · Transketolase

Abbreviations
TK   Transketolase
TDP   Thiamine diphosphate
R5P   Ribose 5-phosphate
X5P   Xylulose 5-phosphate

1 Introduction

Transketolase (TK, EC 2.2.1.1) belongs to a group of thiamine diphosphate-dependent enzymes and catalyzes the reversible transfer of a double-carbon fragment (glycolaldehyde residue) from keto sugars (donor substrates) to aldo

O. N. Solovjeva · I. A. Sevostyanova · V. A. Yurshev ·
V. A. Selivanov · G. A. Kochetov (✉)
A.N. Belozersky Institute of Physico-chemical Biology, Moscow
State University, Moscow 119992, Russia
e-mail: kochetov@genebee.msu

sugars (acceptor substrates). Typical donor substrates of TK are represented by xylulose 5-phosphate (X5P), fructose 6-phosphate, and sedoheptulose 7-phosphate, whereas ribose 5-phosphate (R5P), 3-phosphoglyceraldehyde, and erythrose 4-phosphate act as acceptor substrates [5].

Transketolase *S. cerevisiae* is a dimer of two structurally identical subunits of 74.2 kDa each [21]. A single subunit comprises three domains: N-, or PP-domain (amino acid residues 3–322); intermediate, or Pyr-domain (residues 323–538), and C-domain (residues 539–680). The dimer structure of TK is formed largely via the interaction between PP- and Pyr-domains. The coenzyme, thiamine diphosphate (TDP), interacts with the same domains. TK has two active centers localized to a deep cleft between the subunits. The thiazole ring of TDP is bound to both subunits, essentially via hydrophobic interactions. Only the C2 atom of the thiazole ring, which is the site of donor substrate binding, is accessible to the solvent [10, 12]. The aminopyrimidine ring is found within a hydrophobic pocket formed primarily by residues of aromatic amino acids of the Pyr-domain of one of the subunits. The pyrophosphate group is bound to the PP-domain of the other subunit [12].

Ca^{2+}, which is found within the native holoenzyme, acts as a cofactor, in addition to TDP. Other bivalent cations are also capable of functioning as cofactors of the enzyme [4, 7]. Substitution of one such cation for its functional equivalent exerts no effect on the catalytic activity of TK; nevertheless, the properties of the enzyme change considerably. If Ca^{2+} is replaced by Mg^{2+}, for example, the affinity of TDP for apoTK undergoes a dramatic decrease [8, 9, 15]. The stability of the enzyme is also cation-dependent: it is more stable in the presence of Ca^{2+} than Mg^{2+}. Differences in certain structural characteristics have been reported, as well [1, 3, 14].

Native holoTK contains Ca^{2+} (2 g atom per mol protein, or one cation per each active center) [6]. The ion is involved

in TDP binding and serves as a bridge between the apo-protein and the pyrophosphate group of the coenzyme [16]. However, the number of Me^{2+}-binding sites in the molecule of TK is in excess of two [13]. It would seem that the function of bivalent cations is not limited to their involvement in the coenzyme binding; other experimental data reported in the literature lend support to this assumption [11]. The goal of this work was to study the effect of free Ca^{2+} on the interactions of the substrates with holoTK and the catalytic activity of the enzyme.

2 Materials and Methods

2.1 Materials

TDP, glyceraldehyde phosphate dehydrogenase, NAD^+, R5P, glycyl-glycine, and $CaCl_2$ were purchased from MP Biomedicals (Germany); sodium arsenate and dithiothreitol, from Fluka (Switzerland); Sephadex G-50, from Pharmacia (Sweden). Other commercially available chemicals were of the highest grade.

2.2 Methods

2.2.1 Purification of Apotransketolase

TK was isolated as described previously [18], the preparation was stored at 4 °C in a 50% saturated solution of ammonium sulfate (pH 7.6). The enzyme was homogeneous by SDS/PAGE (specific activity, 12 U/mg). The concentration of TK was determined spectrophotometrically ($A_{1cm}^{1\%}= 14.5$ at 280 nm) [2]. Ammonium sulfate was eliminated by passing apoTK through Sephadex G-50.

2.2.2 Preparation of Holotransketolase

Reconstitution of holoTK from the apoenzyme and coenzyme (100 μM) was performed in 50 mM glycyl-glycine buffer, pH 7.6, in the presence of 2.5 mM $CaCl_2$ for 30 min. In order to eliminate both TDP and Ca^{2+} ions not bound by the protein, the preparation of the reconstituted holoTK was passed through a Sephadex G-50 column. No TDP or Ca^{2+} dissociation from holoTK could be detected in the course of subsequent experiments.

2.2.3 Measurement of Transketolase Activity

TK activity was measured spectrophotometrically (Aminco DW 2000) by the rate of NAD^+ reduction in a coupled system containing glyceraldehyde phosphate dehydrogenase, using X5P and R5P as substrates [5].

2.2.4 Preparation of Xylulose 5-phosphate

X5P sodium salt was prepared enzymatically by a method developed previously; hydroxypyruvate and phosphoglyceraldehyde were used as substrates for TK from baker's yeast [20].

2.2.5 Measurement of Xylulose 5-phopshate and Ribose 5-phosphate Concentrations

Measurement of X5P and R5P concentrations was performed as described previously [19].

#### 2.2.6 Determination of Kinetic Parameters

Kinetic parameters were determined by fitting the dependence of TK reaction rate on the concentration of one of the substrates at a constant concentration of the other substrate. Experimental data were analyzed using the Michaelis equation

$$v = \frac{V \cdot [S]}{[S] + Km} \qquad (1)$$

and the equation

$$v = \frac{V2 + V1 \cdot Km2/[S]}{1 + Km2 / [S] + Km1 \cdot Km2/[S]^2}, \qquad (2)$$

which corresponds to the following tentative reaction scheme:

$$E \xleftrightarrow{Km1} ES1 \xleftrightarrow{Km2} ES2 \rightarrow V2,$$
$$\downarrow$$
$$V1$$
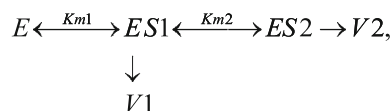
where E designates TK; S, one of the substrates (X5P or R5P); ES1, the enzyme harboring the substrate in one of the active centers; and ES2, the enzyme, in which the second active center is also engaged by the substrate.

2.2.7 Measurement of the Hill Coefficient

In order to determine the Hill coefficient, the experimental data were fitted to the Hill equation

$$\left(v = \frac{Vmax \cdot S^n}{Km + S^n} \right) \qquad (3)$$

All fittings were performed using GNUPLOT, a program that implements the Levenberg-Marquart algorithm for fitting procedure.

3 Results and Discussion

3.1 Effect of Ca²⁺ on Kinetic Characteristics of Transketolase Reaction

Without Ca²⁺ additions to the medium, the dependence of holoTK activity on the concentration of the donor substrate, X5P, is hyperbolic: the experimental points fall within the curve obtained by experimental data fitting according to Eq. (1) (Fig. 1).[1] This indicates that the active centers of the enzyme are equivalent in terms of substrate binding. In the presence of external Ca²⁺, however, the dependence is no longer described by the Michaelis–Menten kinetics, which suggests that the active centers of TK are non-equivalent with respect to X5P (or there is cooperative interaction between them).

Indeed, the experimental points fall within the curve obtained by experimental data fitting according to Eq. (2) (Fig. 2). The values of K_m and V thus determined are listed in Table I, which shows that in the presence of Ca²⁺, the value of K_m, determined with X5P for one of the two active centers of the enzyme (to which we arbitrarily assign number one), decreases, whereas the value of K_m for the other active center (correspondingly, center No. 2) increases by an order of magnitude. The value of the Hill coefficient, determined by fitting the Eq. (3) to the same experimental data, is equal to 0.67 ± 0.02, which indicates negative cooperativity between the centers. Of note, this is paralleled by decrease in the activity of the first center and increase in that of the second one.

The results obtained with the acceptor substrate, R5P, are, in essence very much similar. In the absence of external Ca²⁺, both centers exhibit the same affinities for R5P. Conversely, in the presence of Ca²⁺, the active centers become non-equivalent (or to interact cooperatively), which is manifested as a sharp increase in the affinity for the substrate, determined for the first center, and a decrease of the same extent in the affinity of the second center. These changes are associated, respectively, with decreased activity of the first center and increased activity of the second center (Table 1). The values of the Hill coefficient, determined based on experiments with R5P, are virtually the same as those derived from X5P experiments.

The experimental data obtained indicate that holoTK is catalytically active even in the absence of external Ca²⁺. Note that the active centers of the enzyme are equivalent in both the affinity for the substrates (X5P and R5P) and the catalytic activity. In the presence of free Ca²⁺ (added from an external source), binding of either X5P or R5P induces negative cooperativity, whereas their subsequent catalytic conversion causes the interaction between the centers to
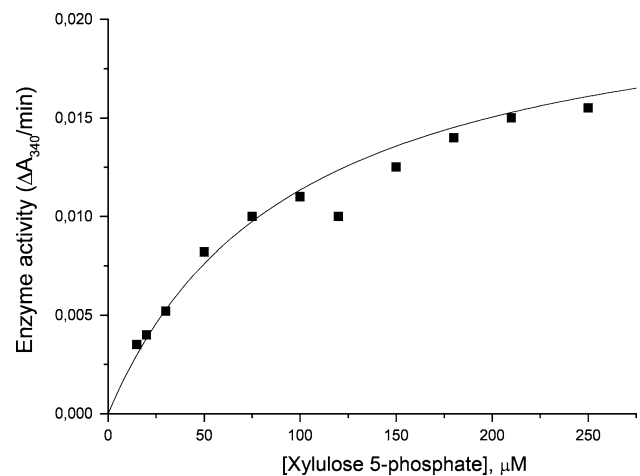
Fig. 1 Effect of X5P concentration on the activity of holoTK in the absence of free Ca²⁺. The concentration of R5P was equal to 1,500 μM. The concentration of the enzyme in the sample was equal to 0.4 μg/ml, Ca²⁺—100 μM. Each point is an experimental value, and the curve is a result of fitting according to Eq. (1)
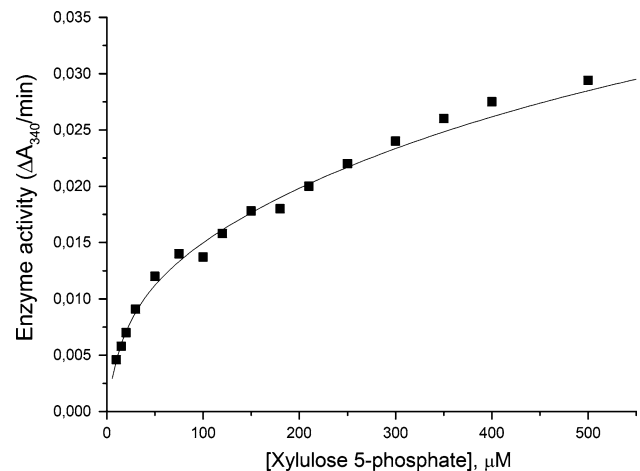
Fig. 2 Effect of X5P concentration on the activity of holoTK in the presence of 100 μM free Ca²⁺. The concentration of R5P was equal to 1,500 μM. The concentration of the enzyme in the sample was equal to 0.4 μg/ml, Ca²⁺—100 μM. Each point is an experimental value, and the curve is a result of fitting according to Eq. (2)

become positively cooperative. The effect of Ca²⁺ is specific, because, addition of Mg²⁺ (instead of Ca²⁺) does not affect kinetic characteristics of TK, in that nonequivalence of the active centers is not manifested (data not shown).

It is not incurious in this connection that Ca²⁺ fails to exert inducing effects on TK from human tissues: in its presence as well as in its absence, either active center exhibits the same affinity for the substrates (both X5P and R5P) (data not shown). Note, however, that human TK contains Mg²⁺ [17], whereas only Ca²⁺ has been identified in the enzyme from *S. cerevisiae* [6].

It should be stressed that the molecule of the reconstructed *S. cerevisiae* holoTK, which was used in our

---

[1] Here, as in Fig. 2, we show data of one reproducible experiment.

Table 1 Kinetic parameters of holotransketolase in the absence and in the presence of 100 μM Ca^{2+}

	Variable substrate	K_m1 (μM)	K_m2 (μM)	V_1 (U/mg)	V_2 (U/mg)
No Ca^{2+}	Xylulose 5-phosphate[a]	75 ± 9		34 ± 2	
Plus Ca^{2+}	Xylulose 5-phosphate[a]	25 ± 6	773 ± 300	15 ± 3	69 ± 17
No Ca^{2+}	Ribose 5-phosphate[b]	159 ± 17		31 ± 3	
Plus Ca^{2+}	Ribose 5-phosphate[b]	7 ± 2	698 ± 223	5 ± 1	62 ± 11

[a] The concentration of X5P was varied in the range 10–800 μM. The concentration of R5P was fixed at 1,500 μM

[b] The concentration of R5P was varied in the range 5–800 μM. The concentration of X5P was fixed at 900 μM

kinetic experiments, harbored one Ca^{2+} per active center (otherwise the enzyme would be inactive). The non-equivalence of the active centers is only induced as a result of addition into the medium of free Ca^{2+}. It is believed that this effect of free Ca^{2+} may result from its binding to peripheral sites distinct from the site involved in TDP binding.

The cooperative effects involving substrate binding to and their conversion by TK, observed in the presence of free Ca^{2+} (added to the medium from an external source), may indicate that this cation performs some regulatory functions. The concentrations of free Ca^{2+}, used in our experiments (100 μM), match those actually measured inside living yeast cells [22].

References

1. Esakova OA, Meshalkina LE, Kochetov GA (2005) Life Sci 78:8–13
2. Heinrich CP, Noack K, Wiss O (1972) Biochem Biophys Res Commun 49:1427–1432
3. Heinrich C, Schmidt D (1973) Experientia 29:1226–1227
4. Heinrich H, Steffen H, Janser P, Wiss O (1972) Eur J Biochem 30:533–541
5. Kochetov GA (1982) Methods Enzym 90:209–223
6. Kochetov GA, Philippov PP (1970) Biochem Biophys Res Commun 38:930–933
7. Kochetov GA, Philippov PP (1970) Dokl Acad Nauk USSR 19:1234–1236
8. Kochetov GA, Meshalkina LE, Usmanov RA (1976) Biochem Biophys Res Commun 69:836–843
9. Kochetov GA, Tikhomirova NK, Philippov PP (1975) Biochem Biophys Res Commun 63:924–930
10. Lindqvist Y, Schneider G, Ermler V, Sundström M (1992) EMBO J 11:2373–2379
11. Meshalkina LE, Kochetov GA (1979) Dokl Acad Nauk USSR 248:1482–1486
12. Nikkola M, Lindqvist Y, Schneider G (1994) J Mol Biol 238:387–404
13. Philippov PP, Tikhomirova NK, Kochetov GA (1978) Physico-Chem Methods of Mol Biol pp 57–64, Moscow State University, Moscow
14. Selivanov VA, Kovina MV, Kochevova NV, Meshalkina LE, Kochetov GA (2004) FEBS Lett 567:270–274
15. Selivanov VA, Kovina MV, Kochevova NV, Meshalkina LE, Kochetov GA (2003) J Mol Catal. B Enzym 26:33–40
16. Schneider G, Lindqvist Y (1998) Biochim Biophys Acta 1385:387–398
17. Schenk G, Duggleby RG, Nixon PF (1998) Int J Biochem Cell Biol 30:1297–1318
18. Soloviева ON (2002) Biochemistry (Moscow) 67:667–671
19. Soloviева ON, Bykova IA, Meshalkina LE, Kovina MV, Kochetov GA (2001) Biochemistry (Moscow) 66:932–936
20. Soloviева ON, Kochetov GA (2008) J Mol Catal B Enzym 54:90–92
21. Sundström M, Lindqvist Y, Schneider G, Hellman U, Ronne H (1993) J Biol Chem 268:24346–24352
22. Yonatt J (1993) Critical Rev Microbiol 19:83–97