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Cleaving of Ketosubstrates by Transketolase and the Nature of the Products Formed

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Abstract—The interaction of transketolase ketosubstrates with the holoenzyme has been studied. On addition of ketosubstrates cleaving both irreversibly (hydroxypyruvate) and reversibly (xylulose 5-phosphate), identical changes in the CD spectrum at 300-360 nm are observed. The changes in this spectral region, as previously shown, are due to the formation of the catalytically active holoenzyme from the apoenzyme and the coenzyme, and the cleavage of ketosubstrates by transketolase. The identity of the changes in transketolase CD spectrum caused by the addition of reversibly or irreversibly cleaving substrates indicates that in the both cases the changes are due to the formation of an intermediate product of the transketolase reaction—a glycolaldehyde residue covalently bound to the coenzyme within the holoenzyme molecule. Usually, in the course of the transferase reaction, the glycolaldehyde residue is transferred to an aldose (acceptor substrate), resulting in the recycling of the holoenzyme free of the glycolaldehyde residue. The removal of the glycolaldehyde residue from the holoenzyme appears to proceed even in the absence of an aldose. However, the glycolaldehyde cannot be found the free state because it condenses with another glycolaldehyde residue formed in the course of the cleavage of another ketosubstrate molecule yielding erythrulose.

Key words: transketolase, circular dichroism, thiamine enzymes

Transketolase (TK) is a thiamine-dependent enzyme. Its cofactors are thiamine diphosphate (ThDP) and bivalent cations such as calcium and magnesium [1]. The enzyme molecule consists of two subunits of equal molecular weight and similar structure [2] and has two active sites of the same activity but different affinity towards the coenzyme [3]. Transketolase catalyzes the reversible transfer of a two-carbon fragment, the glycolaldehyde residue, from a ketose (donor substrate) to an aldose (acceptor substrate). The reaction yields a new aldose and a new ketose. The transketolase reaction is irreversible if hydroxypyruvate (HP) is used as a substrate. Typical donor substrates for transketolase are xylulose 5-phosphate (Xu5P), fructose 6-phosphate, and sedoheptulose 7-phosphate; acceptor substrates are ribose 5-phosphate, glyceraldehyde 3-phosphate (GA3P), erythrose 4-phosphate, and glycolaldehyde.

Abbreviations: TK) transketolase; ThDP) thiamine diphosphate; GAPDH) glyceraldehyde-3-phosphate dehydrogenase; Xu5P) xylulose 5-phosphate; HP) hydroxypyruvate; GA3P) glyceraldehyde-3-phosphate.

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Transketolase is a representative of transferases requiring two substrates for its functioning—ketose and aldose. However, there are indirect data in the literature indicating the possibility of ketose transformation catalyzed by TK in the absence of an aldose [4-6]. In the present work, we have thoroughly investigated this possibility.

MATERIALS AND METHODS

In the work we used enzyme mixture containing triosephosphate isomerase and α-glycerophosphate dehydrogenase, xylulose 5-phosphate, ribose 5-phosphate (sodium salts) from Sigma (USA); ThDP and glycylglycine from Serva (Germany); dithiothreitol from Fluka (Germany); NAD^+, NADH, and GAPDH from Reanal (Hungary). Other chemicals were of domestic production.

Transketolase was isolated from baker's yeast as described in [7] and was stored at 4°C in ammonium sulfate solution (50% saturation). The enzyme was homogeneous by SDS-PAGE and had specific activity 20 U/mg. Before use, the enzyme solution was passed through a Sephadex G-50 column to remove the ammonium sulfate.

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Transketolase concentration was determined spectrophotometrically using $A^{1\%}_{1cm} = 14.5$ at 280 nm [8].

Transketolase activity assay using two-substrate reaction. TK activity was assayed spectrophotometrically at 25°C by the rate of NAD^+ reduction in a coupled system with GAPDH [1]. The reaction mixture (final volume 1 ml) contained: 50 mM glycylglycine, 10 mM sodium arsenate, 0.37 mM NAD^+, 3 U GAPDH, 3.2 mM dithiothreitol, 2.5 mM $CaCl_2$, 0.08 mM ThDP, 0.07 mM Xu5P, and 0.5 mM ribose 5-phosphate, pH 7.6. The reaction was started by the addition of TK. The measurements were made using 1-cm cells in an Aminco DW-2000 spectrophotometer (USA) at 340 nm.

Transketolase activity assay using a single substrate (Xu5P). The activity was assayed as described above except for the absence of aldose (ribose 5-phosphate) in the reaction mixture. Concentrations of TK and Xu5P are given in the figure legends.

GA3P concentration was determined spectrophotometrically by the change in absorbance at 340 nm due to NAD^+ reduction in the presence of GAPDH.

Erythrulose assay. After the complete conversion of Xu5P by TK (in the absence of aldose but in the presence of GAPDH) was achieved, the reaction mixture was treated with perchloric acid. The proteins precipitated were removed by centrifugation, and the supernatant was assayed for erythrulose content using a method described previously [9].

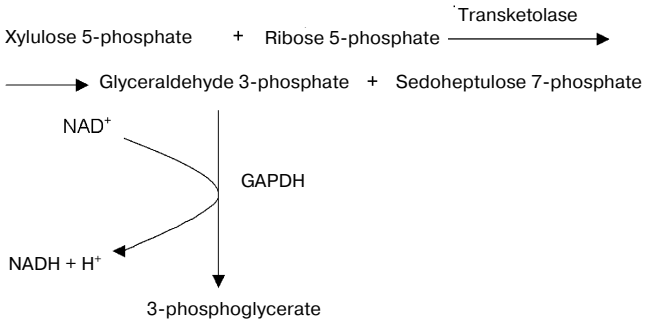
Xylulose 5-phosphate purification. Commercial Xu5P preparations contain impurities of aldoses, mostly ribose 5-phosphate. To remove ribose 5-phosphate, a reaction mixture of the following composition was prepared: 50 mM glycylglycine, pH 7.6, 1 mM sodium arsenate, 3.2 mM dithiothreitol, 1.25 mM NAD^+, 2.5 mM $CaCl_2$, 0.08 mM ThDP, 1 U/ml GAPDH, 3-7 mM Xu5P, and 1.5 U/ml TK. The mixture was incubated at room temperature for 15-20 min, and then the reaction was stopped by the addition of perchloric acid. The efficiency of the removal of aldehyde impurities was estimated by the absence of the initial burst in the kinetic curves obtained in the course of TK activity assay using Xu5P as a substrate without acceptor substrate (see above).

Circular dichroism spectra were recorded in the IBM-PS 1-controlled Mark V dichrograph (Jobin Ivon, France) using 1-cm cells. Data were analyzed using the RDA program. All measurements were performed in 50 mM glycylglycine buffer, pH 7.6, containing 2.5 mM $CaCl_2$ and 4.2 μM TK.

Xylulose 5-phosphate concentration was determined enzymatically by the amount of NADH formed in the presence of GAPDH as a coupling enzyme. The reaction mixture was the same as described for TK activity assay in the two-substrate reaction (see above), but the Xu5P concentration was lowered to 30-100 μM. The concentration of TK was 15-20 μg/ml. The reaction was performed until Xu5P was completely exhausted.

RESULTS AND DISCUSSION

The first product of the two-substrate reaction catalyzed by TK is GA3P if the donor substrate used is Xu5P. The reaction can be monitored spectrophotometrically by the change in absorbance at 340 nm due to NAD^+ reduction in the course of GA3P oxidation catalyzed by GAPDH, which is used as a coupling enzyme.



A typical time dependence of absorbance at 340 nm is shown in Fig. 1, curve *1*. The formation of GA3P from Xu5P was also shown to proceed in the absence of the acceptor substrate in the reaction mixture (curves *2-4* in Fig. 1), although at a lower rate, i.e., about 2% of that of

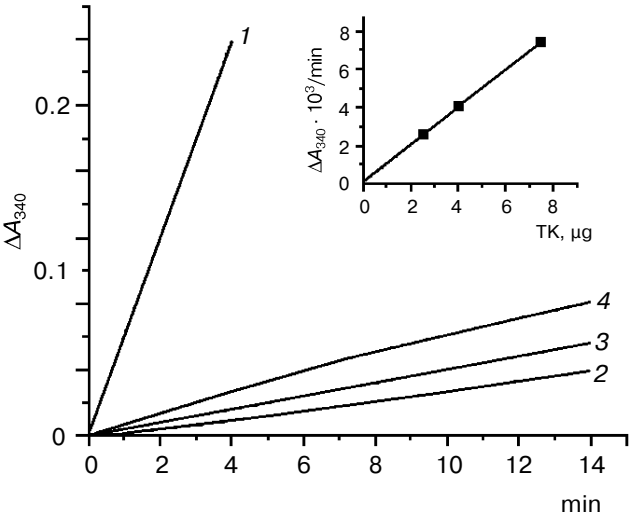


Fig. 1. Change in absorbance at 340 nm during the transketolase reaction: *1*) two substrates, ketose (Xu5P) and aldose (ribose 5-phosphate), were used; *2-4*) only a single substrate, ketose (Xu5P), was used. The reaction mixture (total volume 1 ml) contained: 50 mM glycylglycine, 10 mM sodium arsenate, 0.37 mM NAD^+, 3 U GAPDH, 3.2 mM dithiothreitol, 2.5 mM $CaCl_2$, 80 μM ThDP, 20 μM Xu5P, 0.5 mM ribose-5-phosphate (for the sample with two substrates), and various amounts of TK: 1, 2.5, 4, and 7.5 μg (*1*, *2*, *3*, and *4*, respectively); pH 7.6. The reaction was initiated by the addition of TK. The insert represents the dependence of the single-substrate reaction rate on the TK concentration.

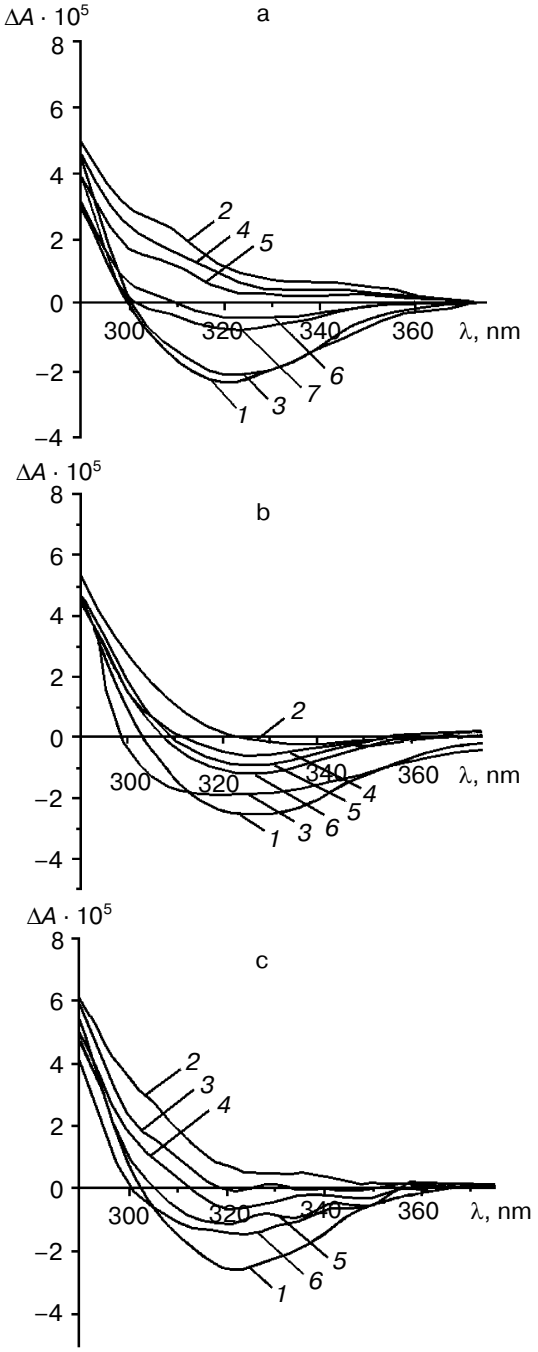


Fig. 2. Near ultraviolet CD spectra of transketolase. The spectra were recorded in 50 mM glycylglycine buffer, pH 7.6, containing 2.5 mM $CaCl_2$. The holoTK concentration was 4.2 μM. a: *1*) holoTK; *2*) holoTK after the addition of 100 μM HP; *3*) same as *2* but after the addition of 1 mM ribose 5-phosphate; *4-7*) same as *2* but the spectra were recorded after 10, 20, 30, and 40 min of incubation with HP, respectively. b: *1*) holoTK; *2*) holoTK after addition 10 μM Xu5P; *3*) same as *2* but after the addition of 1 mM ribose 5-phosphate; *4-6*) same as *2* but the spectra were recorded after 5, 10, and 30 min of incubation with Xu5P, respectively. c: *1*) holoTK; *2*) holoTK after addition 10 μM Xu5P (in the presence of 0.12 mM NADH and the mixture of triosephosphate isomerase and α-glycerophosphate dehydrogenase (5 μg/ml)); *3-6*) same as *2* but the spectra were recorded after 1, 5, 10, and 20 min of incubation, respectively.

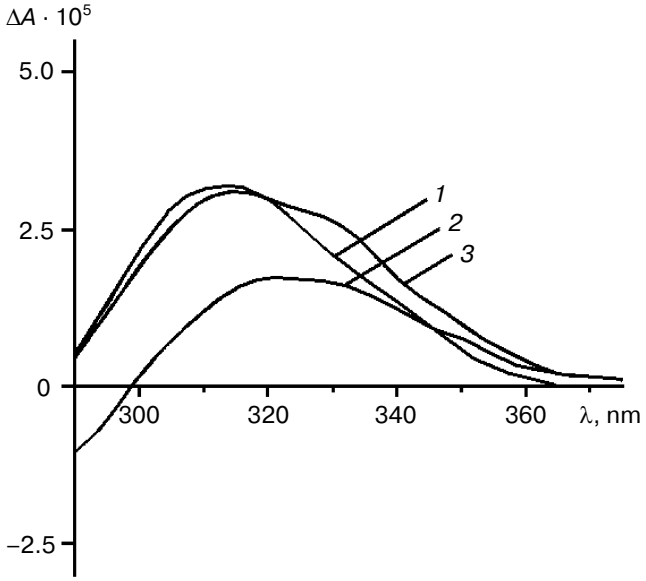


Fig. 3. Near ultraviolet differential CD spectra of transketolase: *1*) (holoTK + HP) – holoTK; *2*) (holoTK + Xu5P) – holoTK; *3*) the same as *2* but in the presence of triosephosphate isomerase, α-glycerophosphate dehydrogenase, and NADH.

the two-substrate reaction. A strict linear dependence between the rate of the single-substrate reaction and the amount of the enzyme added is observed (insert to Fig. 1).

Previously it was shown that the interaction of ThDP with apoTK resulted in the appearance of a new band in the CD spectrum with a maximum at 315-320 nm, this band being absent in the spectra of the initial compounds [10-12]. The intensity of the band strictly correlated to the amount of catalytically active holoenzyme [13, 14] and depended on the substrate concentrations [11, 14]. This allowed the study of the interaction of coenzyme and its analogs with apoTK as well as to separate the investigation of the first and second steps of the TK reaction—the binding and cleavage of the donor substrate and the transfer of the two-carbon fragment to the acceptor substrate, respectively.

Figure 2a (curve *1*) shows the holoTK spectrum. On the addition of HP (the irreversibly cleaving donor substrate), the inversion of the ThDP-induced band at 300-360 nm is observed (curve *2*), this reflecting the first step of the TK reaction—the formation of the intermediate product dihydroxyethyl-ThDP (i.e., a glycolaldehyde residue covalently bound to the coenzyme within the TK molecule). In the second step of the reaction, the glycolaldehyde residue is cleaved from the coenzyme and transferred to the exogenous acceptor substrate (in this case, ribose 5-phosphate), yielding the second product of the reaction (the first was CO_2) and the original holoenzyme. The original spectrum of the holoenzyme is also restored (curve *3* in Fig. 2a).

The holoenzyme spectrum is gradually restored in the absence of the acceptor substrate (curves *4-7* in Fig. 2a). This indicates that the glycolaldehyde residue cleaves from the dihydroxyethyl-ThDP and the original holoenzyme is released, i.e., the single-substrate reaction proceeds.

The results obtained with irreversibly cleaving substrate (HP) were supported in the experiments with reversibly cleaving substrate (Xu5P). Figure 2b demonstrates the holoTK spectrum (curve *1*). After the addition of Xu5P, the ThDP-induced band of the CD spectrum disappears (curve *2*), but the subsequent addition of an aldose restores the band (the original holoenzyme form recycles) (curve *3*). As in the case with HP, the band in the region of 300-360 nm is also restored the absence of aldose, although at a lower rate than in the presence of aldose (curves *4-6* in Fig. 2b). The only difference between the experiments with the two types of substrates was that on the addition of Xu5P to holoTK, the ThDP-induced band disappears (curve *2* in Fig. 2b), while on the addition of HP, its inversion is observed (curve *2* in Fig. 2a). These differences are due to the fact that the first of the two substrates cleaves reversibly, while HP is decarboxylated irreversibly. Thus, in the presence of HP (at sufficiently high concentration) all the holoenzyme is transformed into the complex containing the glycolaldehyde residue, while in the presence of Xu5P some part of the holoTK remains as a complex with uncleaved substrate because of the reversibility of the reaction. Therefore, the differential spectra obtained by subtracting the holoTK spectrum from the spectra of holoTK + HP and holoTK + Xu5P are of identical shape, varying in their amplitudes (Fig. 3, curves *1* and *2*, respectively). If NADH together with the mixture of coupling enzymes (triosephosphate isomerase and α-glycerophosphate dehydrogenase) is added into the sample containing Xu5P, the reaction of Xu5P cleavage becomes irreversible,

Xylulose 5-phosphate decrease and erythrulose formation during the single-substrate reaction catalyzed by transketolase

Xu5P decrease, μmol	Erythrulose formation	
	Expected yield, μmol	Observed yield, μmol
0.7	0.35	0.32
0.21	0.105	0.09

and the inversion of the induced band of CD spectrum is also observed, similar to that in the presence of HP (Fig. 2c, curve *2*). In this case, all the transketolase is converted into the holoenzyme bound to a glycolaldehyde residue (similarly to the experiments with HP), and the quantitative differences in the differential spectra disappear (Fig. 3, curves *1* and *3*). After some time of incubation of the sample containing holoTK + Xu5P in the presence of coupling enzymes (Fig. 2c), the holoenzyme spectrum is restored (curves *3-6*), similarly to that observed in the system containing holoTK + HP (Fig. 2a, curves *4-7*) as well as holoTK + Xu5P in the absence of the coupling enzymes (Fig. 2b, curves *4-6*).

Therefore, similarly to the system with HP, the changes in holoTK spectrum after the addition of Xu5P are due to the formation of the intermediate product of the TK reaction, hydroxyethyl ThDP, with subsequent removal of the glycolaldehyde residue and recycling of the original holoenzyme.

The first product of the single-substrate TK reaction (and also the two-substrate reaction) must be GA3P, if Xu5P is used as a substrate. This is revealed in the exper-

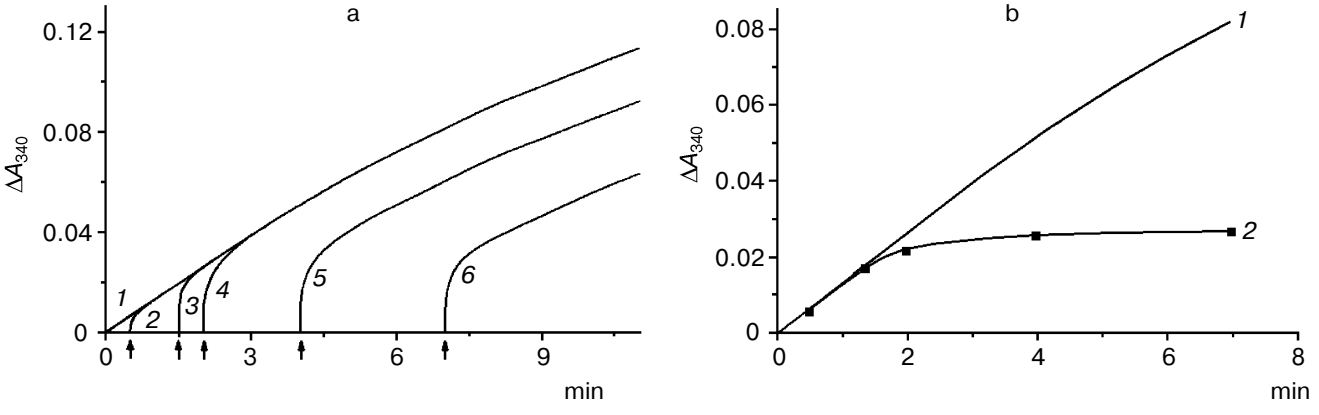


Fig. 4. Kinetics of GA3P accumulation during the single-substrate TK reaction monitored by the change in absorbance at 340 nm. The sample contained 5 μg TK and 70 μM Xu5P. a: *1*) GAPDH was added into the sample before starting of the reaction; *2-6*) GAPDH was added 0.5, 1.5, 2, 4, and 7 min after the beginning of the reaction, respectively (shown by arrows); b: *1*) in the presence of GAPDH (a continuous record was made); *2*) in the absence of GAPDH (after different time intervals, the amount of GA3P accumulated in the reaction mixture was determined as indicated in "Materials and Methods").

iment (see Fig. 1). The second product must be free glycolaldehyde. However, we failed to reveal it in the reaction mixture either chemically or enzymatically (using alcohol dehydrogenase).

Since glycolaldehyde can serve as an acceptor substrate in the TK reaction, it was expected to do the same in the single-substrate reaction, condensing with a glycolaldehyde residue formed in the course of the cleavage of another Xu5P molecule to yield erythrulose. The amount of the erythrulose produced must be half that of the Xu5P subjected to the catalytic transformation. This was found to be true (see table). It cannot be excluded, however, that free glycolaldehyde is not released to the solution but remains bound to the enzyme until its condensation with a glycolaldehyde residue formed from another Xu5P molecule occurs, yielding erythrulose.

Figure 4a demonstrates the kinetics of GA3P accumulation (its amount is equal to the amount of NAD^+ reduced in the course of the reaction)—the first product of the single-substrate reaction catalyzed by TK. The coupling enzyme (GAPDH) was added into the reaction mixture before the reaction was started (curve *1*) or some time later (shown by arrows) after the beginning of the reaction (curves *2*-*6*). In the second case, the initial burst of the accumulation curves of the reaction product (GA3P) reflects its amount produced before GAPDH addition.

As seen from Fig. 4b, the initial rate of the single-substrate reaction does not depend on the presence of the coupling enzyme. Then, in the absence of GAPDH, the rate of the reaction gradually slows to zero (Fig. 4b, curve *2*). This cannot be due to either TK inactivation or its inhibition by reaction products since the addition of GAPDH (Fig. 4a, curves *5* and *6*) after complete blocking of the reaction (Fig. 4b, curve *2*) restores the product accumulation rate up to that observed in the sample containing GAPDH from the very beginning, and maintains it virtually constant for a considerable time (Fig. 4b, curve *1*). The reason for the slowing down and cessation of the reaction is the fact that in the absence of GAPDH the single-substrate reaction is reversible and proceeds until the equilibrium is achieved between the initial substrate (Xu5P) and the reaction products (GA3P and erythrulose). In the presence of GAPDH, the reaction becomes irreversible and proceeds until the substrate is completely exhausted.

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