ELSEVIER

Journal of Molecular Catalysis B: Enzymatic 11 (2001) 771–776

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

www.elsevier.com/locate/molcatb

Enzymatic syntheses of ketoses: study and modification of the substrate specificity of the transketolase from *Saccharomyces cerevisiae*

L. Hecquet [a,*], C. Demuynck [a], G. Schneider [b], J. Bolte [a]

[a] *Université Blaise Pascal, Departement de Chime, UMR 6504 (SEESIB), 63177 Aubière Cedex, France*

[b] *Division of Molecular Structural Biology, Department of Medical Biochemistry and Biophysics, Doktorsringen, Karolinska Institutet, S-171 77 Stockholm, Sweden*

Abstract

Transketolase (TK) is a useful catalyst for ketose syntheses. The first part of this paper reports a convenient and easy method to synthesise 4-deoxy-D-fructose-6-phosphate, potential inhibitor of sugar metabolism. TK used in synthetic purposes is the enzyme from *Saccharomyces cerevisae*, which is commercially available, or the enzyme from spinach leaves which we obtained as a crude extract. But these sources are expensive or give small quantities of the enzyme. In order to obtain larger amounts of enzyme, we use TK overexpressed in *S. cerevisiae*. The three-dimensional structure being known, the study and modification of the substrate specificity of this enzyme can be investigated by site-directed mutagenesis. In the second part of this paper, our study shows that Asp 477 is involved in determining the stereospecificity towards C2 hydroxyl group of the acceptor substrate.

Keywords: Ketose; Transketolase; Substrate specificity; Stereospecificity; Site-directed mutagenesis

1. Introduction

Transketolase (TK) (EC 2.2.1.1) is essential for the metabolism in all living cells. This enzyme is intracellular and is implicated in the pentose phosphate pathway. The enzyme requires two cofactors for activity: thiamine pyrophosphate (TPP) and Mg^{2+}. TK reversibly transfers the C1–C2 ketol unit from D-xylulose-5-phosphate to an aldose phosphate to generate a new ketose phosphate and D-glyceraldehyde-3-phosphate (Scheme 1).

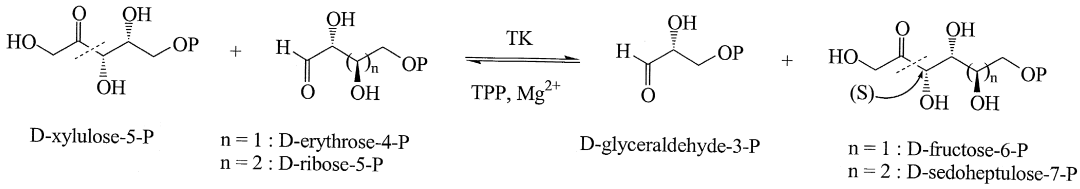
The new stereocenter is in the (*S*) configuration. TK leads to a ketose with D-*threo* configuration. Indeed, the stereospecificity of TK towards the 2-hydroxyaldehyde with (*R*) stereochemistry yields to a ketose with (4*R*) configuration. These characteristics make TK useful for the preparation of chiral synthons [1–8]. For synthetic purposes, the ketol group can be generated by β-hydroxypyruvic acid or by L-erythrulose [9].

2. Ketose syntheses catalysed by TK

β-Hydroxypyruvic acid is the most widely-used donor substrate. The decarboxylation of the β-hy-

* Corresponding author.

1381-1177/01/$ - see front matter
PII: S1381-1177(00)00035-7

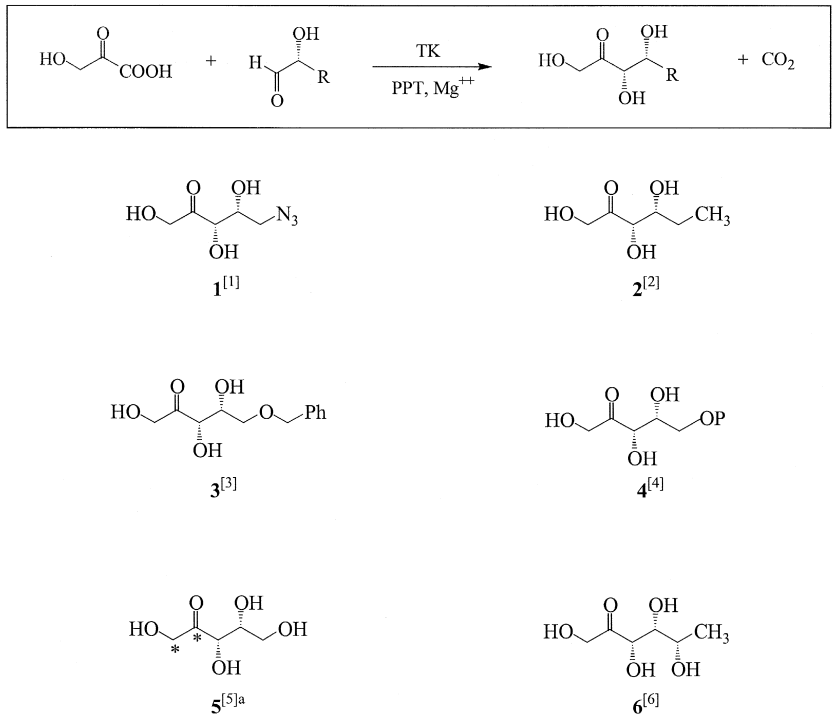


Scheme 1. Reactions catalysed in vivo by TK.

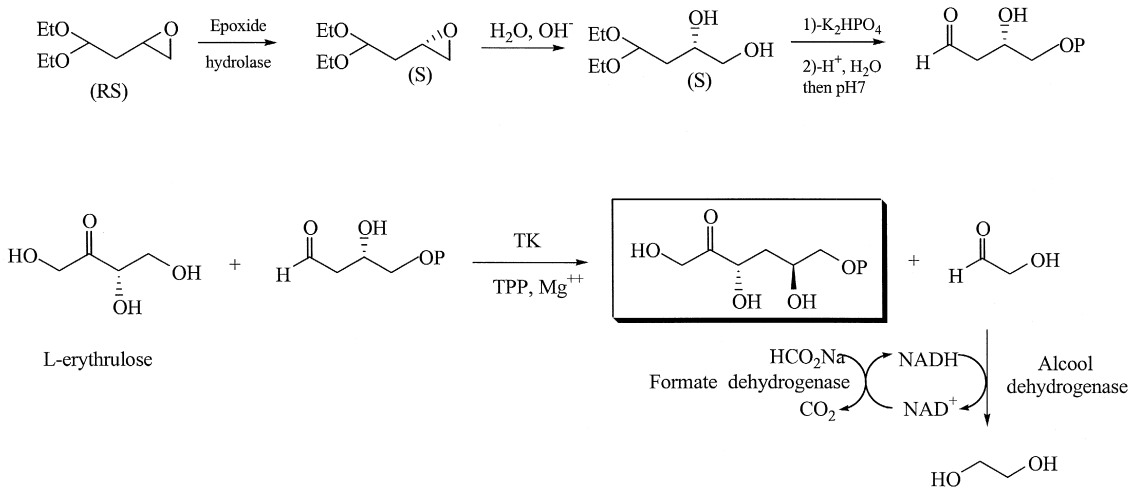
droxypyruvic acid and subsequent loss of carbon dioxide from the reaction mixture render the overall condensation reaction irreversible (Scheme 2).

A wide range of aldehydes are ketol acceptors and this reaction has been used to synthesise various ketoses. The azidosugar **1** has been obtained with good yield as a precursor of corresponding pyrrolidine, a very effective glycosidase inhibitor [1]. An optically active hydroxyketone **2** has been used to accede to a pheromone, brevicomine [2]. A convenient method has been developed to synthesise 5-*O*-benzylxylulose **3** and this reaction can be carried out on a gram scale [3]. Recently, D-xylulose-5-phosphate **4** has been prepared according to a one-pot procedure starting from fructose-1,6-bisphosphate as precursor [4]. Our group has developed a convenient method to prepare [1,2-^{13}C]-labeled-D-xylulose **5** from [2,3-^{13}C] hydroxypyruvic acid [5]. We have also described a biological route to the natural 6-deoxy-L-sorbose [6] precursor of a furanone with caramel-like flavour used in the food industry.

The drawback of β-hydroxypyruvic acid is its instability. The ketol group can be generated by another donor substrate L-erythrulose [9]. This com-



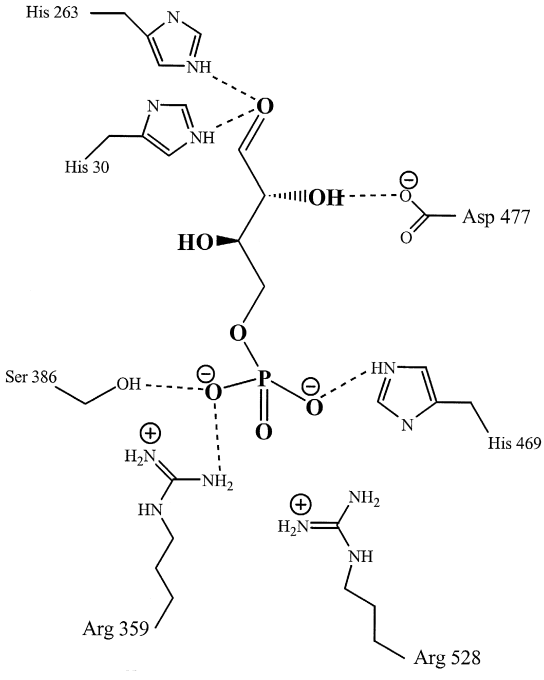
Scheme 2. Examples of ketose syntheses with hydroxypyruvic acid as donor substrate.



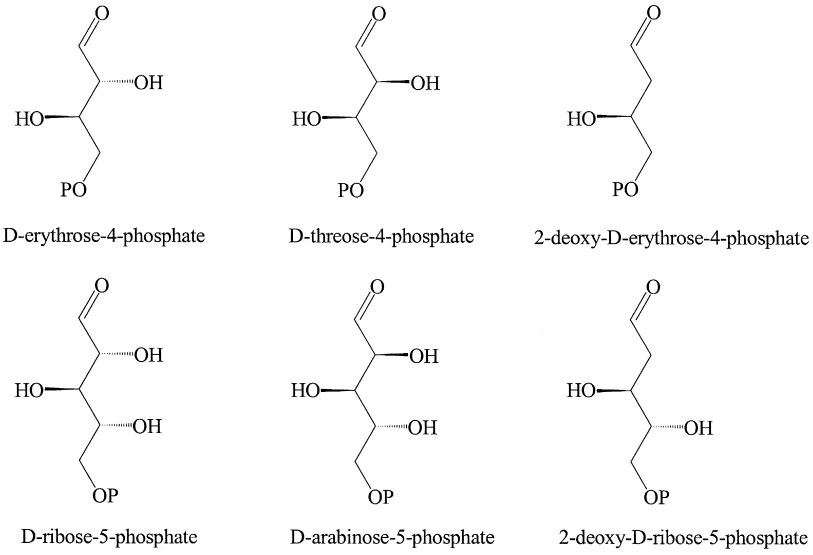
Scheme 3. Synthesis of 4-deoxy-D-fructose-6-phosphate with L-erythrulose as a donor substrate.

pound is a good substrate for TK with a K_m of 5 mM instead of 33 mM for β-hydroxypyruvic acid. This reaction is however reversible: the glycolaldehyde released is also a substrate of TK. In order to shift the equilibrium to the synthesis of the new ketose, the glycolaldehyde is reduced in situ into glycol in the presence of yeast alcohol dehydrogenase and NADH (Scheme 3). This reaction provides a convenient and cheap TK assay method and can also be useful for synthesis purpose as described later. We have developed a convenient method using L-erythrulose as a donor substrate, to synthesise 4-deoxy-D-fructose-6-phosphate [10]. It is a substrate of hexokinase and phosphoglucose isomerase leading to 4-deoxy-D-fructose-6-phosphate and 4-deoxy-D-glucose-6-phosphate [8]. In this respect, it should appear to be a very useful tool to explore the metabolism of fructose. This compound could be a potential inhibitor of fructose-1,6-bisphosphate aldolase and could interfere with sugar metabolism.

In order to prepare 4-deoxy-D-fructose-6-phosphate, the acceptor substrate must be 2-deoxy-D-erythrose-4-phosphate in an optically pure form because its enantiomer is an inhibitor of the reaction. Our strategy to synthesise this compound is based on the opening of this enantiopure epoxide. To prepare the epoxide in the (*S*) configuration, we used the epoxide hydrolase resolution of racemic epoxide [11–13]. Opening of this epoxide by inorganic phosphate in basic conditions followed by acidic hydrolysis of the acetal moiety gives enantiopure aldehyde phosphate. The TK condensation is performed using L-erythrulose as donor substrate. The equilibrium is shifted to the synthesis of the ketose in the presence of yeast alcohol dehydrogenase and the cofactor NADH is recycled using formate/formate dehydro-



Scheme 4. Active site of TK with D-erythrose-4-phosphate.



Scheme 5. Acceptor substrates in the TK reaction used in this study.

genase. This method is actually easy to perform at least for small-scale synthesis. This new procedure is also very convenient from an economical point of view, comparing to the costs of the necessary reagents with the one of β-hydroxypyruvic acid generally used.

3. Study and modification of the active site of TK from *Saccharomyces cerevisiae*

TK used for synthetic purposes is the enzyme from *S. cerevisiae* which is commercially available or the enzyme from spinach leaves which we obtained as a crude extract [5–8]. Now, as is often the case when an enzyme presents a great interest in organic synthesis, the gene has been overexpressed in a micro-organism. Recombinant TK from *Escherichia coli* [3,4] has been used for various ketose syntheses in the literature. At the present time, we use TK overexpressed in *S. cerevisiae* [14]. Since the three-dimensional structure of this enzyme is known [15], we can study the active site in order to modify the substrate specificity by site-directed mutagenesis.

From the three-dimensional crystal structure, TK from *S. cerevisiae* has two identical subunits consisting of 680 amino acid residues each of 74 kDa. Each subunit contains an identical active site situated at the subunit–subunit interface. Each active site contains a binding site for each of the two cofactors, TPP and Mg^{2+}.

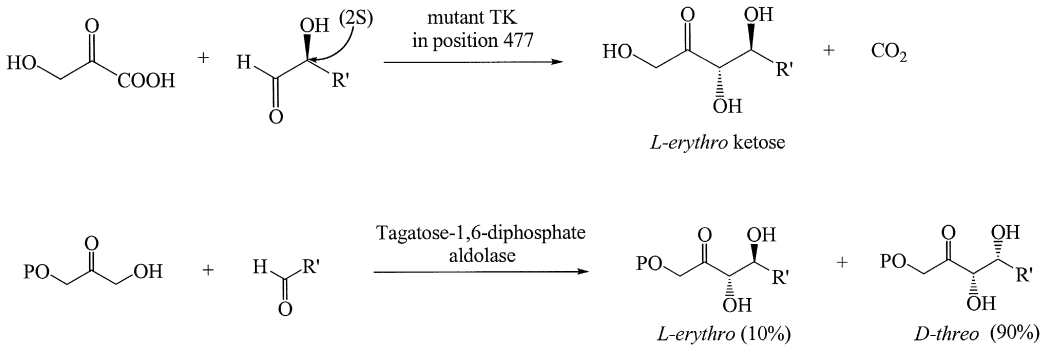
TK has also been crystallised in the presence of the natural acceptor substrate, D-erythrose-4-phosphate [16]. The crystal structure analysis of the enzyme–acceptor substrate complex gives insight into the interactions with the substrate in the active site (Scheme 4).

The inner part of the substrate channel contains several His residues, the middle part has one Ser and

Table 1
Kinetic parameters for wild type and mutant TK[a]

	K_{cat}/K_m (M^{-1} s^{-1})	
	Wild type	Asp 477Ala
D-ribose-5-phosphate	3.1×10^5	3.5×10^2
D-erythrose-4-phosphate	1.7×10^6	4.5×10^3
2-Deoxy-D-ribose-5-phosphate	1.2×10^2	1.1×10^2
2-Deoxy-erythrose-4-phosphate	3.0×10^3	1.8×10^3
D-arabinose-5-phosphate	1.1×10^2	0.5×10^2
D-threose-4-phosphate	0.7×10^2	0.5×10^2

[a] In all cases xylulose-5-phosphate was used as the donor substrate.



Scheme 6. Synthesis of L-erythro ketose catalysed by a mutant TK and by tagatose-1,6-bisphosphate aldolase.

the outer part two Arg and one His. Our goal is to prove the significance of these interactions for the specificity and for the catalytic efficiency. Several of the acceptor substrates used in this study were not commercially available and were obtained by organic syntheses [16].

The first results concern the role of Asp 477 in the stereospecificity towards the hydroxyl group on C2 of the acceptor substrate, we have determined kinetic parameters of wild type and mutant TK (Asp 477 is replaced by Ala) using natural acceptor substrates with (2*R*) configuration, analogues with inverted stereochemistry (2*S*) and 2-deoxy analogues (Scheme 5) (Table 1). The 2-deoxyaldehydes were included in this series, since removal of the hydroxyl group should be compared to the modification of the protein by the Ala mutation. The ratio k_{cat}/K_m is used to assess the overall efficiency and specificity of enzyme action. In all cases D-xylulose-5-phosphate is used as the donor substrate.

(2*S*) and 2-deoxy substrates give similar k_{cat}/K_m values. We can observe that the wild type TK shows a severe decrease of k_{cat}/K_m for (2*S*) and 2-deoxy substrates. In fact, the inversion of the stereocenter or removal of the hydroxyl group disrupts the hydrogen bond of the side chain of Asp 477. This result confirms the stereospecificity of wild type TK towards the hydroxyl group of C2 of the acceptor substrate. When repeating the same measurements on the mutant TK, k_{cat}/K_m values for natural substrates and analogues are in the range of the values for wild type TK with analogues. The results indicate that the replacement of Asp 477 by an Ala leads to the loss of the stereospecificity and strongly decreases the efficiency of TK. Asp 477 is involved in determining the stereospecificity of TK [17].

4. Conclusion

According to these results, the key role of Asp 477 for the stereospecificity of TK could offer the most interesting prospects for synthetic purposes. We could investigate a mutation at 477 position of TK allowing inversion of the stereospecificity while maintaining the efficiency.

Such a mutant TK might lead to L-erythro ketoses (Scheme 6). This is important because among the enzymes catalysing C–C bond formation, only the tagatose-1,6-bisphosphate aldolase could lead to ketoses with this stereochemistry but this enzyme is not particularly stereoselective.

References

[1] T. Ziegler, A. Straub, F. Effenberger, Angew. Chem., Int. Ed. Engl. 27 (1988) 716.
[2] D.C. Myles, P.J. Andrulis, G.M. Whitesides, Tetrahedron Lett. 32 (1991) 4835.
[3] K.G. Morris, M.E.B. Smith, N.J. Turner, Tetrahedron: Asymmetry 7 (1996) 2185.
[4] F.T. Zimmermann, A. Schneider, U. Schörken, G.A. Sprenger, W.D. Fessner, Tetrahedron: Asymmetry 10 (1999) 1643.
[5] J. Bolte, C. Demuynck, L. Hecquet, H. Samaki, Carbohydr. Res. 206 (1990) 79.
[6] L. Hecquet, J. Bolte, C. Demuynck, Tetrahedron 50 (1994) 8677.

[7] L. Hecquet, J. Bolte, C. Demuynck, Tetrahedron 52 (1996) 8223.
[8] C. Andre, J. Bolte, C. Demuynck, Tetrahedron: Asymmetry 9 (1998) 3737.
[9] L. Hecquet, J. Bolte, C. Demuynck, Biosci., Biotechnol., Biochem. 57 (1993) 2174.
[10] C. Guerard, V. Alphand, A. Archelas, C. Demuynck, L. Hecquet, R. Furstoss, J. Bolte, Eur. J. Org. Chem. (1999) in press.
[11] A. Archelas, R. Furstoss, Trends Biotechnol. 16 (1998) 108.
[12] A. Archelas, R. Furstoss, in: W.-D. Fessner (Ed.), Biocatalysis. From Discovery to Application, 200, 160.
[13] I.V.J. Archer, Tetrahedron 53 (1997) 15617.
[14] C. Wikner, L. Meshalkina, U. Nilsson, M. Nikkola, Y. Lindqvist, M. Sundström, G. Schneider, J. Biol. Chem. 23 (1994) 32144.
[15] M. Nikkola, Y. Lindqvist, G. Schneider, J. Mol. Biol. 238 (1997) 387.
[16] U. Nilsson, L. Meshalkina, Y. Lindqvist, G. Schneider, J. Biol. Chem. 272 (1997) 1864.
[17] U. Nilsson, L. Hecquet, T. Gefflaut, C. Guerard, G. Schneider, FEBS Lett. 424 (1998) 48.