Asp^{477} is a determinant of the enantioselectivity in yeast transketolase

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Abstract The conserved residue Asp^{477} in yeast transketolase is located in the substrate channel of the enzyme and forms a hydrogen bond with the C2-hydroxyl group of the acceptor substrate. The significance of this interaction for the recognition of the preferred acceptor substrates, D-α-hydroxyaldehydes was investigated by site-directed mutagenesis. In the wild-type enzyme the k_{cat}/K_M values are by three to four orders of magnitude lower for 2-deoxyaldoses or substrates with L-configuration at the C2-atom. In the Asp^{477}Ala mutant, the k_{cat}/K_M values for D-α-hydroxyaldehydes are decreased by a thousandfold, while the k_{cat}/K_M values for substrates with L-configuration or 2-deoxyaldoses are similar to wild-type enzyme. These results indicate that Asp^{477} is involved in determining the enantioselectivity of transketolase.

Key words: Thiamine diphosphate; Substrate specificity; Site-directed mutagenesis; Enzyme mechanism; Enantioselectivity

1. Introduction

Transketolase (EC 2.2.1.1) is a thiamine diphosphate (ThDP) dependent enzyme composed of two identical subunits [1] with a molecular mass of 74.2 kDa each [2]. The thiamine diphosphate molecule binds in a deep cleft at the interface between the two subunits and is completely inaccessible from the outer solution, except for the C2-atom of the thiazolium ring to which the donor substrate binds covalently. The fold of the enzyme, protein-ThDP interactions and the catalytic mechanism of transketolase have been investigated by crystallography [3–6] and site-directed mutagenesis of various conserved amino acids in the cofactor binding and active site of the yeast [7–10] and the human enzyme [11,12].

Transketolase catalyses cleavage and formation of carbon–carbon bonds, transferring a ketol moiety from a ketosugar to an aldosugar (Fig. 1). The enzyme has high specificity for donor ketoses with D-*threo* (C3-L, C4-D) configuration and for α-hydroxylated acceptor aldoses with C2-D configuration [13,14]. Transketolase has received much attention as a potential tool in organic chemistry. Of particular interest is the use of hydroxypyruvate as donor substrate in preparative synthesis, since this reaction is practically irreversible due to the formation of CO_2. Wild-type transketolase has been used for recovering L-α-hydroxyaldehydes from racemic aldehyde substrates [15] as well as for the stereospecific synthesis of D-2-hydroxyaldehydes [14], D-[1,2-^{13}C2]xylulose [16] and other ketoses [17]. The enzyme has also been used in more complex syntheses, for instance the stereospecific synthesis of the beetle pheromone (+)-exo-brevicomin [14] and the glycosidase inhibitor fagomine [18]. It is therefore of interest to reveal the structural features responsible for the stereospecific recognition of donor and acceptor substrates, with the objective to provide the basis for attempts to modify the substrate specificity of this enzyme.

The three-dimensional structure of the acceptor substrate erythrose-4-P bound to the enzyme and the characterisation of residues involved in the interactions with the substrate in the active site, provided insight into the binding of substrates to transketolase [6,9]. The crystal structure analysis of the enzyme–acceptor substrate complex [6] suggested that the conserved residue Asp^{477}, located in the substrate channel of the enzyme, might be responsible for the observed enantioselectivity of the transketolase reaction. In this study we have probed this proposal by investigating the stereospecificity of wild-type transketolase and a point-mutant, where Asp^{477} had been replaced by alanine. The analysis provides evidence that Asp^{477} indeed is an important determinant of enantioselectivity in transketolase.

2. Materials and methods

2.1. Chemicals

Thiamine diphosphate, D-xylulose-5-phosphate, D-ribose-5-phosphate, 2-deoxy-ribose-5-phosphate, D-erythrose-4-phosphate and glyceraldehyde-3-phosphate dehydrogenase were obtained from Sigma. All other reagents used were of the purest grade commercially available.

2.2. Synthesis of substrates

Several of the acceptor substrates used in this study were not commercially available and were synthesized as follows.

2.2.1. Synthesis of D-arabinose-5-phosphate. The procedure was based on that described by Bednarski and colleagues [19]. To a solution of 1 g phosphoenolpyruvate potassium salt in water (7.5 ml, pH 7.0) in a 25-ml Erlenmeyer flask containing a magnetic stirring bar were added: 0.981 g D-arabinose, 0.11 g adenosine triphosphate (ATP) and 0.19 g $MgCl_2 \cdot 6H_2O$. The pH was adjusted to pH 7.6 with 1 N NaOH. The solution was degassed with N_2 for 30 min and 0.1 ml of mercaptoethanol, hexokinase (900 U) and pyruvate kinase (45 U) were added. After 4 days, 900 U of hexokinase were added to the reaction mixture. After 3 more days, 900 U of hexokinase and 45 U of pyruvate kinase and 0.45 g of $MgCl_2 \cdot 6H_2O$ were added. After an additional 5 days, 0.4 g of $BaCl_2 \cdot 2H_2O$ was added (in 0.1-g portions dissolved in approximately 1 ml of water) to precipitate remaining ATP and phosphate. After each addition, the mixture was filtered through celite and each filter cake was washed with approx-

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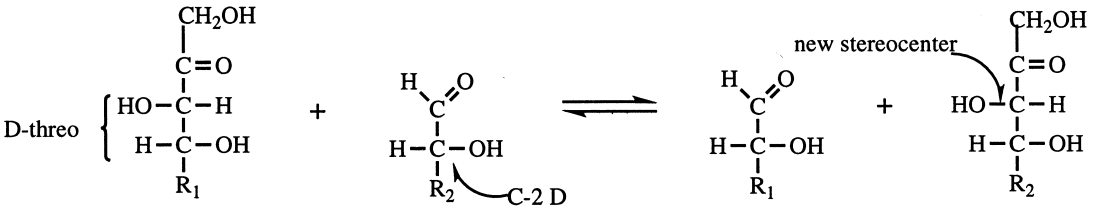

Fig. 1. Scheme of the transketolase reaction.

imately 5 ml of water. To the combined filtrates (30 ml) 1.3 g of $BaCl_2 \cdot 2H_2O$ and then 90 ml of ethanol were added and the mixture was left at 4°C overnight. The precipitate was collected by filtration, washed with 10 ml of ethanol/water (3:1, v/v) and dried in vacuo over P_2O_5 to give 1.95 g of the barium salt (82% yield, 75% purity). The purity of this compound was determined by nuclear magnetic resonance (NMR). Phosphoenolpyruvate and pyruvate are present by approximately 15% and 10%, respectively (these compounds do not interfere with the enzyme assay). The barium salt of D-arabinofuranose-5-phosphate was characterized by ^{13}C NMR spectra. Major anomer form: ^{13}C NMR (100 MHz, D_2O). δ 103.85 (C1); 83.87 (C4); 78.41 (C2); 85.07 (C3); 66.24 (C5). Minor anomer form: ^{13}C NMR (100 MHz, D_2O). δ 97.99 (C1), 78.70 (C4), 76.93 (C2), 83.20 (C3), 67.31 (C5). Before use, the Ba^{2+} salt of the sugar phosphate was desalted by treatment with Dowex 50WX8 (H^+ form).

2.2.2. Synthesis of 2-deoxy-D,L-erythrose-4-phosphate. To a solution of 7.4 g K_2HPO_4 in 60 ml of water was added 2.6 g 3,4-epoxybutanal diethylacetal obtained according to a procedure described previously [20]. The mixture was heated under reflux for 24 h. During this time the mixture turned dark yellow. The solution was extracted with 3×30 ml of ether. To the aqueous layer was added a solution of 15 g barium acetate in 20 ml of water. The pH of the mixture was adjusted to 8.0 and the formed precipitate was removed by centrifugation. Four volumes of ethanol were added and the resulting solution was kept at 4°C overnight. The formed precipitate was recovered by centrifugation and dried in vacuo to obtain the barium salt of 3,4-dihydroxybutanal-4-phosphate diethylacetal (2.2 g, 35% yield, 64% purity, the residual 36% is potassium acetate). The compound was characterized by ^1H and ^{13}C NMR spectra. ^1H NMR (400 MHz, D_2O, HOD = 4.9ppm) δ 1.25 (m, 6H, $CH(OCH_2\underline{CH_3})_2$), 1.85 (m, 2H, $\underline{CH_2}CH(OEt)_2$), 3.75 (m, 7H, $CH(OCH_2\underline{CH_3})_2$, $\underline{CH}(OEt)_2$, $\underline{CH_2}OPO_3Ba$), 3.95 (m, 1H, $\underline{CH}OH$). ^{13}C NMR (100 MHz, D_2O): δ 107.10 (C6), 39.52 (C3), 65.77 (C5), 70.32 (C2, $J = 7.04$ Hz), 70.95 (C1, $J = 4.83$ Hz), 103.67 (C4). Before use, the Ba^{2+} salt was desalted by treatment with Dowex 50WX8 (H^+ form).

2.2.3. Synthesis of D-threose-4-phosphate. I_2 (3.12 g) was added to a solution of tribenzyl phosphite (4.77 g) in 50 ml CH_2Cl_2 at −20°C. After 20 min, the clear colorless solution was allowed to warm to 25°C and was subsequently added dropwise to a solution of (4*R*,5*R*)-2,2-dimethyl-2,3-dioxolane-4,5-dimethanol (2 g), synthesized according to Batsanov et al. [21], and pyridine (4 ml) in 50 ml CH_2Cl_2 at −20°C. The solution was then allowed to warm to 25°C, filtered and concentrated under reduced pressure. The residue was diluted with diethylether (100 ml) and water (25 ml). The organic layer was washed with aqueous 100 ml 0.3 M $KHSO_4$, 50 ml H_2O, aqueous saturated 50 ml $NaHCO_3$ (50 ml), brine (50 ml) and dried over $MgSO_4$. After concentration, the crude material was purified by flash-chromatography (eluent, CH_2Cl_2-MeOH, 98:2, v/v) to give 2.2 g of [(4*S*,5*R*)-(2,2-dimethyl-5-hydroxymethyl-1,3-dioxolan-4-yl)]-methyl dibenzyl phosphate as a colorless oil (42%): ^1H NMR (400 MHz, $CDCl_3$): δ 7.35 (10H, m), 5.05 (4H, m), 4.15–4.0 (3H, m), 3.90 (1H, m), 3.75–3.55 (2H, m), 3.02 (1H, s), 1.40 (3H, s), 1.36 (3H, s).

To a mixture of this compound (1 g), dimethylsulfoxide (4.5 ml) and triethylamine (1.7 ml) in 4 ml CH_2Cl_2 sulfur trioxide-pyridine (1.9 g) at 0°C was added. After 1 h at room temperature, the solution was diluted with diethylether (25 ml), washed with water (2×15 ml), dried over $MgSO_4$ and concentrated under reduced pressure and under high vacuum. The residue was diluted with anhydrous 50 ml MeOH and 0.5 g *p*-toluene sulfonic acid was added. After 2 h at 25°C, 200 μl triethylamine was added and the solution concentrated under reduced pressure. The residue was diluted with 25 ml EtOAc, washed with 25 ml water, 25 ml aqueous saturated $NaHCO_3$, 25 ml brine and dried over $MgSO_4$. After concentration, the crude material was purified by flash-chromatography (eluent CH_2Cl_2-MeOH, 95:5) to give 0.45 g of [(2*S*,3*S*)-(2,3-dihydroxy-4-dimethoxybut-1-yl)]dibenzyl phosphate as a pale yellow oil (45%): ^1H NMR (400 MHz, $CDCl_3$): δ 7.35 (10H, m), 5.05 (4H, m), 4.38 (1H, d), 4.10 (2H, m), 3.98 (1H, m), 3.57 (1H, m), 3.44 (3H, s), 3.42 (3H, s), 3.37 (1H, d), 3.08 (1H, d); ^{13}C NMR (100 MHz, $CDCl_3$): δ 135.7, 136.6, 128.1, 104.7, 69.8, 69.5, 69.4, 69.0, 68.9, 68.8, 68.7, 56.1, 54.6.

To a solution of 0.36 g of this compound in 10 ml methanol 5% Pd/C (50 mg) was added. Hydrogenolysis was performed at 25°C using a balloon. The reaction was complete after 30 min. The catalyst was removed by filtration. Cyclohexylamine (171 μl) was then added and the solvent evaporated. The residue was washed with 10 ml anhydrous diethylether and dried under high vacuum for 24 h to give 360 mg of [(2*S*,3*S*)-(2,3-dihydroxy-4-dimethoxybut-1-yl)] dicyclohexylammonium phosphate as a white solid (96%): ^1H NMR (400 MHz, CD_3OD): δ 5.37 (8H, s), 4.65 (1H, d), 4.1 (3H, m), 3.85 (1H, m), 3.60 (6H, s), 3.22 (2H, m), 2.25 (4H, m), 2.01 (4H, m), 1.85 (2H, m), 1.55 (8H, m), 1.40 (2H, m); ^{13}C NMR (100MHz, CD_3OD): δ 106.0, 71.6, 71.5, 67.2, 67.1, 56.6, 54.2, 51.5, 32.4, 26.3, 25.8.

To a solution of 70 mg of this compound in 0.5 ml water Dowex 50WX8 (H^+ form, 0.5 ml) was added. The mixture was smoothly stirred for 5 min and the resin was separated by filtration and washed with 0.5 ml water. The combined aqueous solutions were incubated for 24 h at 25°C to give a solution of D-threose-4-phosphate which is totally hydrated together with 2 Eq. of MeOH. ^1H NMR (400 MHz, H_2O+D_2O): δ 5.05 (1H, d), 4.17–4.0 (4H, m); ^{13}C NMR (100 MHz, H_2O+D_2O): δ 92.1, 75.3, 71.9, 69.5.

The ^1H-NMR and ^{13}C-NMR spectra were determined on a Bruker 400-MHz instrument.

2.3. Protein expression and purification

H402×pTKL1 yeast cells carrying plasmids with wild-type [7] or $Asp^{477}Ala$ mutant [6] genes were cultured in leucine deficient medium to obtain a high copy number of the plasmid. Culture conditions were as described [7]. The protocol for protein purification developed by

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

	Wild-type		$D^{477}A$	
	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})
D-Ribose-5-P	46	3.1×10^5	0.8	3.5×10^2
D-Erythrose-4-P	69	1.7×10^6	1.4	4.5×10^3
2-Deoxy-D-ribose-5-P	0.3	1.2×10^2	0.4	1.1×10^2
2-Deoxy-erythrose-4-P	4.9	3.0×10^3	1.3	1.8×10^3
D-Arabinose-5-P	0.4	1.1×10^2	0.05	0.5×10^2
D-Threose-4-P	0.3	0.7×10^2	0.07	0.5×10^2

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

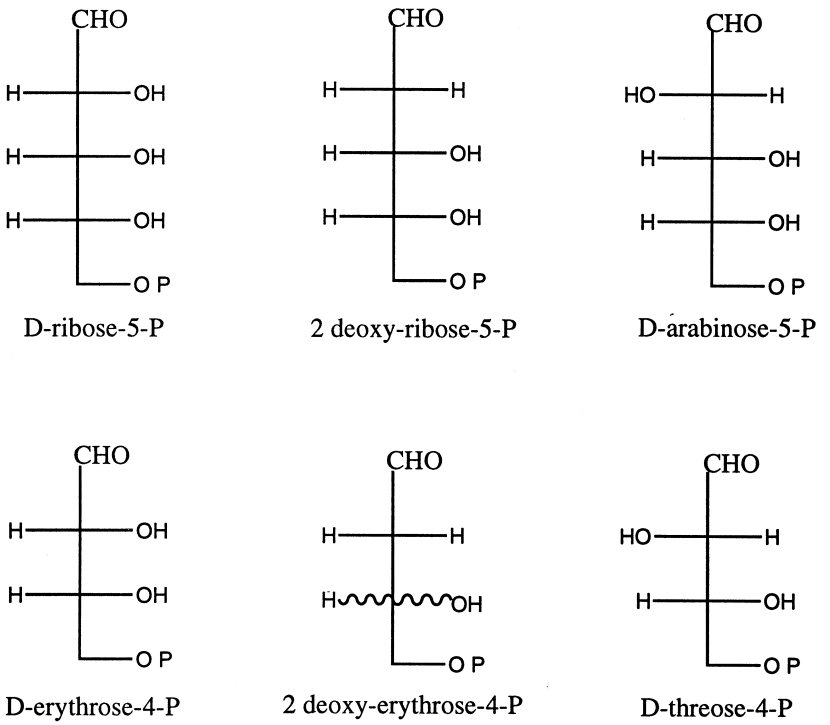

Fig. 2. Acceptor substrates in the transketolase reaction used in this study.

Wikner et al. [7] was used to purify wild-type transketolase and the mutant protein to homogeneity as judged by SDS-PAGE analysis. The protein concentration was determined spectrophotometrically by using the absorption coefficient $A^{1\%}_{280nm} = 14.5$ [22].

2.4. Kinetic measurements

The activity assay was based on a coupled system with glyceraldehyde-3-P dehydrogenase and NAD^+ [23]. The kinetic steady-state parameters were determined from initial rate measurements in 50 mM glycylglycine buffer, pH 7.6. In all assays, the concentration of ThDP was 100 μM and that of $MgCl_2$ was 2.5 mM. Xylulose-5-P, present in large excess, was used as donor substrate, and the concentration of the acceptor substrate in question was varied over 0.1 to 10 times the K_M value. The measurements were carried out in duplicate at 25°C. The data were analyzed by non-linear regression analysis with the program ULTRAFIT (Biosoft) to obtain K_M and V_{max} values and standard errors.

3. Results and discussion

In order to investigate whether Asp^{477} is involved in the recognition of the α-hydroxyl group of the acceptor substrate, we have determined the steady state kinetic parameters of wild-type and Asp^{477}Ala mutant transketolase, using a series of acceptor substrates with different stereochemistry at the C2 position (Fig. 2). The 2-deoxyaldehydes were included in this series, since removal of the hydroxyl group should be comparable to the modification of the protein by the Asp^{477}Ala mutation.

The steady state parameters (Table 1) show that in the wild-type enzyme the presence of a hydroxyl group at the C2 carbon of the acceptor substrate with C2-D configuration is required for high catalytic efficiency. Inversion of the stereochemistry of this carbon atom resulted in a decrease of the k_{cat}/K_M values by approximately three to four orders of magnitude. A similar effect was found when acceptor substrates without a hydroxyl group at this position were used (Table 1). This strongly indicates that the stereochemistry of the C2 hydroxyl group of the acceptor substrate is highly important for the catalytic efficiency of the transketolase reaction.

Replacement of Asp^{477} by alanine results in a significant decrease in the catalytic activity and the k_{cat}/K_M values for the natural acceptor substrates ribose-5-P and erythrose-4-P, while the k_{cat} and k_{cat}/K_M values for the 2-deoxy- and C2-L substrates were comparable to those for the wild-type enzyme. Quantitatively, the change in k_{cat}/K_M for the natural substrates in the mutant is of the same order of magnitude as the difference in the k_{cat}/K_M values in wild-type transketolase between acceptor substrates with C2-D stereochemistry on the one hand and substrates without the α-hydroxyl group on the other.

These results are consistent with the mode of acceptor substrate binding in the active site of transketolase [6] and provide further evidence that Asp^{477} is involved in the enantioselectivity of the transketolase reaction. The acceptor substrate interacts through a number of specific hydrogen bonds with residues in the active site channel (Fig. 3). The conserved residues Arg^{359}, Arg^{528}, His^{469} and Ser^{386} at the entrance of this channel form hydrogen bonds to the phosphate group of the substrate. The C1 oxygen atom of the aldose interacts with the side chains of His^{30} and His^{263}, whereas the C2 hydroxyl group is within hydrogen bonding distance of the side chain of Asp^{477}. As expected, 2-deoxyaldoses are not very good substrates for the enzyme, since this hydrogen bond cannot be formed. In a similar manner, inversion of the stereocenter at the C2-carbon disrupts the hydrogen bond of the side chain of Asp^{477} to the C2 hydroxyl group of the aldose consistent with the observed decrease in specificity of the enzyme as evidenced by the k_{cat}/K_M values. The decrease in enantioselectivity in the Asp^{477} mutant confirms the proposed role of this residue in recognition of the proper stereochemistry at the C2 carbon of the aldose. Since this residue cannot form this hydrogen bond to the 2-deoxy and C2-L aldoses,

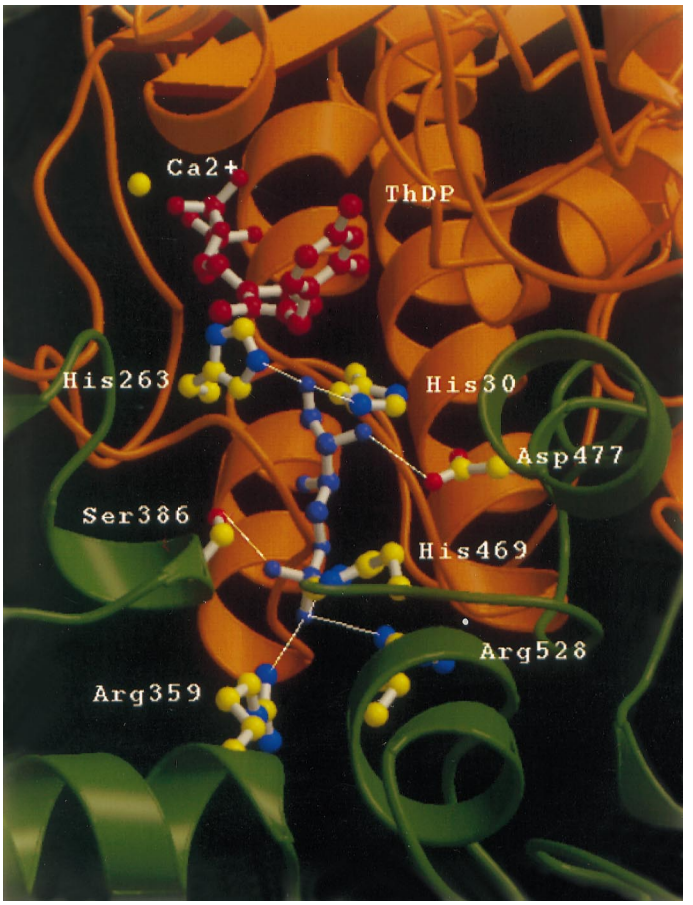

Fig. 3. View of the active site of transketolase with bound acceptor substrate erythrose-4-phosphate (shown in light blue). The two subunits are shown in different colours. Hydrogen bonds are indicated by white lines. The figure was generated with MOLSCRIPT [24].

one expects that the kinetic parameters for these substrates are similar in wild-type and mutant enzyme and indeed, this was observed.

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