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Four types of threonine aldolases: Similarities and differences in kinetics/thermodynamics

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Abstract

Four types of threonine aldolases (TAs) with different stereospecificities were tested on the aldol synthesis of phenylserine (PS) starting from benzaldehyde and glycine under kinetic and thermodynamic control. At short time of reaction the chosen enzymes show different stereoselectivities (L-*syn*, L-*anti*, D-*syn*) compared to the reaction at equilibrium where *syn*-PS is obtained as the major product (*d.e.* ~20%) for all types of TAs. A new aspect of the catalytic mechanism involved and a sight on the relative energy barriers for the possible rate-determining steps were obtained, based on data from the ^{13}C-label distribution between components at the conditions close to equilibrium.

Keywords: Threonine aldolase; Aldol reaction; Phenylserine; Enzyme catalysis; Lyases

1. Introduction

The potential outcome of a chemical reaction is usually influenced by two factors: the relative stability of the products (thermodynamic factors) and the rate of product formations (kinetic factors). The productivity of the enzymatic aldol reaction, when two stereocentres are formed, is limited by the position of the equilibrium and by the reaction rates leading to the formation of the enantiomers [1]. Herein, these factors and their influence on product distribution in threonine aldolase (TA) catalyzed reactions will be discussed.

TAs (EC 4.1.2.5) are pyridoxal-5′-phosphate (PLP) dependent enzymes, which physiologically catalyze the reversible cleavage of threonine into glycine and acetaldehyde [2–4]. In the reverse reaction, threonine derivatives can be formed from glycine with different acceptor aldehydes. A large variety of aliphatic and aromatic aldehydes are converted with complete stereocontrol at the α-carbon, but low specificity at the β-carbon [5–7].

The synthesis of optically pure β-hydroxy-α-amino acids, which are potential chiral building blocks for the synthesis of biologically active molecules (e.g. antibiotics [8,9] and others [10,11]), has for many years been an important synthetic target and several approaches were made [12–14]. The TA-catalyzed synthesis of β-hydroxy-α-amino acids has recently attracted great attention [15–18]. Products were obtained with high enantio-, but low diastereoselectivities. It was noticed that a high diastereomeric ratio can be obtained in a kinetically controlled reaction under conditions far from equilibrium, however, yield is limited [5]. Recently we have reported that yield and selectivity strongly depend on the reaction conditions and an enzymatic procedure was established using DTA from *Alcaligenes xylosoxidans*. This lead to high enantio- and diastereoselectivity in a kinetically controlled reaction, whereas LTA from *Pseudomonas putida* gave only moderate selectivity [19].

Threonine aldolases are divided into two types according to their specificity at the α-carbon in the cleavage reaction of threonine: L- and D-specific threonine aldolases (Fig. 1) [20,21]. With respect to the β-carbon, L-specific threonine aldolases can further be divided into three sub-types: L-threonine aldolase (LTA), which preferably cleaves L-threonine (L-Thr); L-*allo*-threonine aldolase (L*allo*TA), which cleaves L-*allo*-threonine (L-*allo*-Thr);

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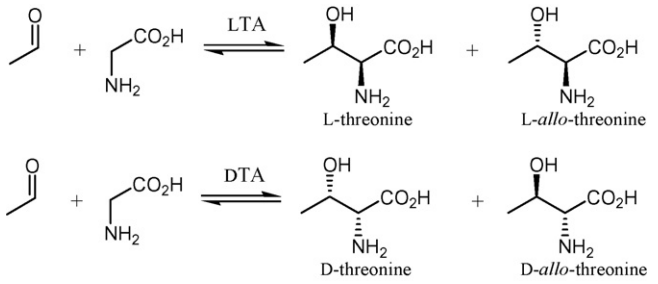


Fig. 1. Threonine aldolase catalysed synthesis of L- and D-threonine.

and L-*low specificity* threonine aldolase (L*low*TA) which accepts both L-Thr and L-*allo*-Thr as substrates. Regarding the D-series only D-*low specificity* threonine aldolase (DTA) could so far be found in nature.

Threonine aldolases are ubiquitous in nature. Genes encoding TA have been found in plants [22], vertebrates [23], several bacteria, yeast [24] and fungi. Comparatively few of them have been overexpressed and tested as catalysts for biotransformations [25–32]. We cloned and overexpressed members of all four known types of threonine aldolases. The recombinant enzymes were examined for the aldol synthesis and retro-aldol cleavage of PS. In this manuscript the similarities and differences in the behavior of all TAs in the kinetically and thermodynamically controlled reactions as well as the reasons for the low diastereoselectivities are discussed. New aspects about the mechanism involved in TA-catalysis are proposed based on the data from ^{13}C distribution experiments.

2. Experimental

2.1. General

All reagents and solvents were obtained from commercial sources and appropriately purified, if necessary. ^{1}H and ^{13}C NMR spectra were recorded on a Varian INOVA 500 (^{1}H 499.82 MHz, ^{13}C 125.69 MHz) using the residual peaks of D_2O (^{1}H: δ 4.79) or DMSO*d6 (^{1}H: δ 2.5, ^{13}C δ 40.2) as references. H_2O/D_2O-NMR samples were directly taken from the aqueous solution, diluted with D_2O (1:1) and recorded using H_2O presaturation [33]. Analytical HPLC was carried out with a Hewlett Packard Series 1100 HPLC using a G1315A diode array detector. A Purospher® STAR RP18 (250 mm, 5 μm) column was used for analysis. Phenylserine isomers were determined by HPLC after derivatization with *ortho*-phthaldialdehyde/2-mercaptoethanol (OPA/MCE, achiral derivatization, *d.e.* determination) and *ortho*-phthaldialdehyde/*N*-acetyl cysteine (OPA/NAC, chiral derivatization, *e.e.* determination) [34,35]. DL-*anti*-PS and DL-*syn*-1,2-^{13}C-PS were synthesized as reference materials using published methods starting from 1,2-^{13}C-glycine [36].

2.2. Bacterial strains and expression constructs

The strains were obtained from the German collection of microorganisms and cell cultures (DSMZ) or isolated from nature and cultivation conditions were used as recommended.

Table 1
Bacterial and yeast strains, type of expressed proteins, construction primers, gene size and protein molecular weight

Enzyme	NCBI accession	Organism	Construction primers	Gene size (bp)	Protein MW (Da)
L*allo*TA	AE000512	*Thermatoga maritima* 3109	5′-atgatcgatctcaggtccgacac-3′ 5′-tcaggagaattttctgaagagtttttcg-3′	1032	37,574
L*allo*TA	D87890	*Aeromonas jandaei* 7311	5′-atgcgctatatcgatttacgaag-3′ 5′-tcacgccccgagatactcggtaaag-3′	1017	36,290
L*allo*TA	AF011922	*Pseudomonas aeruginosa* BT530, BT1510, BT1531	5′-atgcctgtcatcgacctgcgcagcg-3′ 5′-ttatgagcgacgaaaggccgcgaag-3′	1005	35,470
LTA	AE004953	*P. aeruginosa* BT530, BT1510, BT1531	5′-atgaccgatcacacccaacagttcg-3′ 5′-tcaggcgcccatcaccaggcggctgt-3′	1041	38,200
L*low*TA	L28739	*Saccharomyces cerevisiae*	5′-atgactgaattcgaattgcctcc-3′ 5′-tcagtatttgtaggtttttatttcgcgg-3′	1164	42,800
L*low*TA	CP000031	*Silicibacter pomeroyi* 15171	5′-atgttctttgcctctgacaactc-3′ 5′-tcagagcagtgacaggaagcggtc-3′	1038	37,348
L*low*TA	BX640433	*Bordetella parapertussis* 13415	5′-atgatcgatctgcgcagcgatac-3′ 5′-tcagcgcgcggccgcgcaatg-3′	1020	35,662
L*low*TA	BX640448	*Bordetella bronchiseptica* 13414	5′-atgatcgatctgcgcagcgatac-3′ 5′-tcagcgcgcggccgcgcaat-3′	1020	35,662
PSaldolase	–	*Pseudomonas fluorescencs*	5′-atgaacggtgaaacaagcagacc-3′ 5′-ctatcgttcttgtgtgcggtcag-3′	1074	38,320
DHAA	AB075600	*Paracoccus denitrificans* 413	5′-atgaatgcgaaaacggatttctccgg-3′ 5′-tcagtagccctttccgcgcgccgag-3′	1164	41,633
D*low*TA	NC006569	*S. pomeroyi* 15171	5′-atgaaagacatgaccaatctgga-3′ 5′-tcaataggccttgccgcgggccg-3′	1164	41,810

The genomic DNAs from bacteria and yeast were isolated according to the established protocols [37]. The threonine aldolase genes were cloned by PCR from the genomic DNA with primers according to Table 1. PCR amplification was performed in a 50 μL reaction mixture, containing 1 μL of DNA template, 0.5 μM of the corresponding forward and reverse primers, 2 mM of each dNTPs, 10 μL 5× HF Phusion buffer, 0.5 μL Phusion polymerase (Finnzyme) and rest is double distilled H_2O. For the amplification an initial denaturation step at 98 °C for 30 s was followed by 30 cycles of 98 °C, 10 s; 65 °C, 20 s; and 72 °C for 1 min 45 s for denaturing, annealing and elongation, respectively. After the final elongation step reaction proceeded for 5 min at 72 °C. The products of the expected size were purified on gel and cloned into the pEamTA plasmid [38]. NdeI and HindIII digestion was used to confirm the correct gene insert, the positive colonies were selected. Correct plasmids of each type were retransformed into *Escherichia coli* BL21AI (Invitrogen) for protein expression.

2.3. Overexpression of TAs

E. coli BL21AI cells containing pEamTA-TA plasmids were cultivated on 2xTY media supplemented with 0.1 mg/mL ampicilline. 100 mL overnight cultures in 300 mL flasks were inoculated with single colonies and grown at 37 °C with shaking. The 330 mL main cultures in baffled 1000 mL flasks were inoculated with 3 mL of the preculture and grown at 37 °C for approximately 4 h to an OD_{600} of 1.5. Temperature was then lowered to 28 °C and cultures were induced with 0.1 mM of IPTG. Cultivation was continued over night and cells harvested the next day by the centrifugation for 15 min at 4500 × *g*. After resuspension of the pellets in 0.1 M sodium phosphate buffer (pH 7) the cells were disrupted by ultrasonic treatment. The crude lysate was cleared by centrifugation at 20,000 × *g* for 1 h and the supernatant (cell free extract, CFE) was used for enzymatic synthesis without further purification.

2.4. Molecular mass determination

The molecular mass of the expressed proteins was determined by SDS-PAGE (12%) electrophoresis with the low molecular weight standard LMW (Fermentas) as reference.

2.5. Activity assay

The TA-catalyzed retro-aldol reaction of threonine or *allo*-threonine to produce glycine and acetaldehyde was spectrophotometrically monitored by the decrease of NADH absorbance at 340 nm in a 1 cm light path cuvette using a coupled enzymatic reaction where acetaldehyde was reduced to ethanol by yeast alcohol dehydrogenase (ADH). The assay mixture contained D- or L-threonine, D-*allo*- or L-*allo*-threonine (50 mM), KH_2PO_4 (50 mM, pH 8), PLP (50 μM), $MnCl_2$ (only for DTA, 50 μM), NADH (200 μM), ADH (30 U, Sigma) and 50 μL of diluted cell-free extract in a final volume of 1.5 mL. The reactions were started by the addition of the diluted cell-free extract. One unit of the enzyme is the amount of enzyme that causes the formation 1 μmol of NAD^+ per minute at room temperature (24 °C) under above described conditions. The activity of TA with respect to cleavage of *syn*- and *anti*-PS was measured with benzaldehyde dehydrogenase (BALD). The amount of released PS was monitored on behalf of increased NADH absorbance at 340 nm.

2.6. General procedure for the aldol synthesis of PS

To a solution of TA CFE (0.6 U/μmol for Thr), PLP (13 ng, 50 nmol) and $MnCl_2$ (for DTA, 6 ng, 50 nmol) in 1 mL buffer (KH_2PO_4 50 mM, pH 8.0) benzaldehyde (10 μL, 0.1 mmol) and glycine (75 mg, 1.0 mmol) were added. The reaction mixture was stirred at room temperature till reaction reached equilibrium. Samples were taken during the course of reaction, diluted 50 times and analyzed by HPLC and NMR. HPLC: OPA/MCE: buffer KH_2PO_4 (50 mM, pH 8.0)/CH_3CN = 71/29, 0.75 mL/min, t_{syn} = 11 min, t_{anti} = 14.5 min; OPA/NAC: buffer KH_2PO_4 (50 mM, pH 8.0)/CH_3CN = 79/21, 0.75 mL/min, $t_{D\text{-}syn}$ = 11.5 min, $t_{L\text{-}syn}$ = 15.4 min, $t_{D\text{-}anti}$ = 15.5 min, $t_{L\text{-}syn}$ = 21.4 min; NMR data was consistent with those reported [39].

2.7. General procedure for the retro-aldol cleavage of anti-PS

To a solution of TA CFE (0.6 U/μmol Thr), PLP (13 ng, 50 nmol) and $MnCl_2$ (for DTA, 6 ng, 50 nmol) in 1 mL buffer (KH_2PO_4, 50 mM, pH 8.0) L/D-*anti*-PS (40 mg, 0.2 mmol) and glycine (67 mg, 0.9 mmol) were added. The reaction mixture was stirred at rt till reaction reached equilibrium. Samples were taken during course of reaction, diluted in 50 times and analyzed by HPLC.

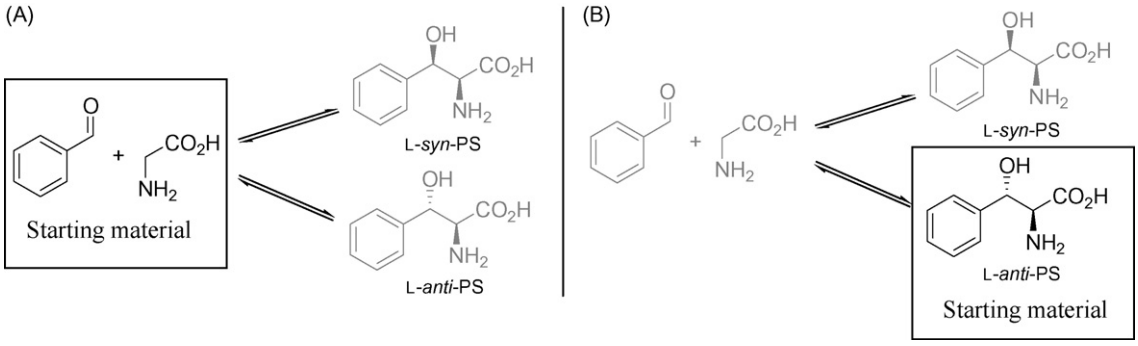
2.8. General procedure for the retro-aldol cleavage of syn-^{13}C-PS

To a solution of L/D-*syn*-^{13}C-PS (8.7 mg, 48 μmol), L/D-*anti*-PS (5.8 mg, 32 μmol), PLP (13 ng, 50 nmol), $MnCl_2$ (for DTA, 6 ng, 50 nmol), benzaldehyde (1.2 μL, 10 μmol) and glycine (35 mg, 0.46 mmol) in 1 mL buffer (KH_2PO_4 in D_2O, 50 mM, pH 8.0) TA CFE (0.3 U/μmol Thr) was added. Reactions were carried out in a NMR tube. NMR data was consistent with those reported for unlabeled compounds [39]. ^{13}C-*syn*-PS, ^{13}C-*anti*-PS and ^{13}C-glycine appeared as satellites from both side of the uncoupled signal.

3. Results and discussion

3.1. Cloning and overexpression of TAs

Four types of threonine aldolase were found in the Genebank (NCBI) by sequence comparison. Genes of L*allo*TA from *Aeromonas jandaei*, *Pseudomonas aeruginosa* and *Thermotoga maritima*, LTA from *P. aeruginosa*, *low specificity* LTA from *Saccharomyces cerevisiae*, *Bordetella parapertussis*, *Bordetella bronchiseptica*, *Silicibacter pomeroyi*, L-phenylserine aldolase



Scheme 1. L-Threonine aldolase-catalyzed (A) aldol synthesis starting from benzaldehyde and glycine and (B) retro-aldol cleavage of L-*anti*-PS.

from *Pseudomonas fluorescence*, D-hydroxyaspartate aldolase from *Paraccocus dinitrificans*; *low specificity* DTA from *S. pomeroyi* were cloned by PCR, A-tailed and ligated into the Eam1105I site of pEamTA vector.

The pEamTA-plasmids contain the TA-genes under control of a *tac* promoter and the LacI repressor, which control the protein expression level. TA expression was induced by 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) in *E. coli* BL21AI cells containing the plasmid. Upon induction the recombinant cells produced moderate to large amount of TA. The induced proteins migrated as a 36–42 kDa bands on a SDS-polyacrylamide gel, in accordance with the predicted amino acid sequence.

3.2. Kinetics and thermodynamics in the TA-catalyzed reactions

TAs were found to accept a wide range of aldehydes as acceptors and only glycine as a donor [5,19]. We chose the synthesis of PS starting from glycine and benzaldehyde as a model reaction to investigate the ability of the new overexpressed aldolases to catalyze the aldol condensation. As it was shown before, extending the time of the reaction catalyzed by TAs increases yields but reduces diastereoselectivity due to the reverse reaction [5,17]. Thus, stereo-enriched products can be obtained in a kinetic mode of reaction (short reaction time). Partial overcoming of the thermodynamic limitation was achieved

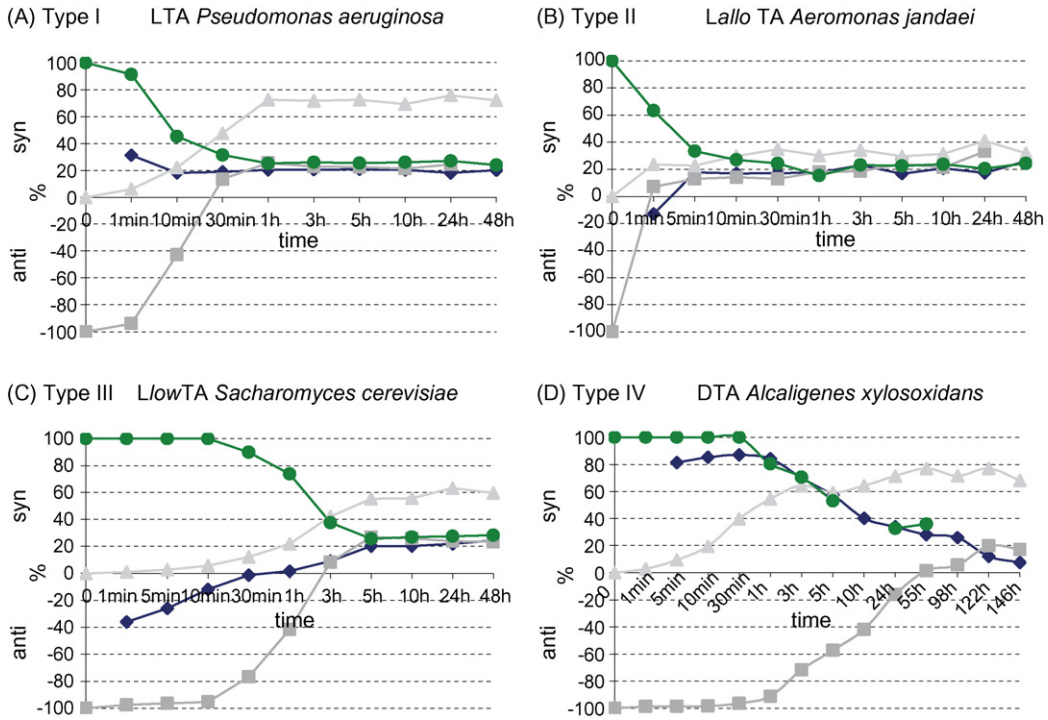


Fig. 2. Change of diastereomeric excess for aldol (♦) and retro-aldol (■, *anti*; ●, *syn*) reactions and yields in aldol reactions (▲) for different threonine aldolases. Conditions of the aldol reaction: 1-mL solution containing glycine (1 M), benzaldehyde (100 mM), PLP (50 μM) at 25 °C, pH 8; the retro-aldol reaction: glycine (0.9 M), L/D-*anti*-PS (0.2 M), PLP (50 μM), TA (0.6 U/μmol Thr) at 25 °C, pH 8; (A) Type I, LTA *Pseudomonas aeruginosa*; (B) Type II, L*allo*TA *Aeromonas jandaei*; (C) Type III, L *low specificity* TA *Saccharomyces cerevisiae*; (D) Type IV, D *low specificity* TA *Alcaligenes xylosoxidans*, $MnCl_2$ (50 μM); *e.e.* >99% for all reactions; all results obtained by HPLC. To make the time course clearly visible the time scale was chosen arbitrarily.

by using an excess of glycine to shift the equilibrium to the aldol side. Detailed investigations of the aldol reactions for all four types of TAs might give answers to important questions about the mechanism of catalysis and elucidate the peculiarity of each type of enzyme. Following the time course of the reactions we monitored the changes of diastereomeric excess versus yield and determined the equilibrium point for the interconversion of both diastereoisomers. Two recombinant TAs of each type from different organisms were tested on the aldol reaction starting from benzaldehyde and glycine (Scheme 1A) and two retro-aldol reactions starting from *anti*-PS (Scheme 1B) or *syn*-PS. The point where aldol and retro-aldol reactions reached the same ratio of diastereomers was defined as equilibrium point.

The detailed analysis of both reactions showed that the four types of threonine aldolases catalyze the interconversion in different ways. Reactions with LTA give higher *d.e.* (>30%) with *syn*-PS as the major isomer under kinetic control (reaction time 1 min) compared to thermodynamic control (reaction time > 30 min) and at the equilibrium *d.e.* is 21% (*syn*) (Fig. 2A, Table 2, Type I). For reactions catalyzed by L*allo*TA epimerization proceeds very fast and PS was obtained with a *d.e.* of 20% (*syn*) after equilibrium was reached (Fig. 2B, Table 2, Type II). On the other hand, for reactions catalyzed by L *low specificity* TA equilibrium was reached only after 5 h (Fig. 2C, Table 2, Type III). Surprisingly, rather high *anti*-selectivity – however low yield – was obtained in a kinetically controlled reaction for L*allo*TA and L*low*TA. All investigated types of L-specific TA gave the same mixture of diastereomers (*d.e.* ≈20%, *syn*) when the reactions are under thermodynamic control. High *syn* selectivity was obtained for the reaction catalyzed by D *low specificity* TA from *A. xylosoxidans* (optimized conditions: *d.e.* ~98%, *syn*; yield 79%) [19]. Detailed investigation of this reaction showed that D-*syn*-PS is obtained with *d.e.* more then 95% and good yield in a kinetically controlled fashion. Thermodynamic equilibrium for the reaction was reached after 5 days with 17% *d.e.* (*syn*) (Fig. 2D, Table 2, Type IV).

Thus, *syn*-PS as a thermodynamically more stable product is formed under equilibrium conditions with a 60:40 (*syn*:*anti*) ratio for all types of TA. Reaction rates for the formation of *syn*-PS and *anti*-PS depend on the catalyst used and therefore different specificities are obtained in a kinetic mode of the reaction. There are two possibilities to affect the diastereoselectivity and yield of the product of the aldol reaction. One method involves the shifting of the thermodynamic equilibrium to the aldol side, which might need additional steps and reagents [1]. Another way is to find a catalyst which has a high energy barrier for the *syn*/*anti* epimerization and was able to produce optically pure compounds with high yield under kinetically controlled conditions. DTA from *A. xylosoxidans* is a good example for this possibility.

3.3. Energy profile of the TA catalyzed aldol/retro-aldol reactions

The mechanism for the retro-aldol reactions catalyzed by TA has already been formulated [40,41]. Based on this knowledge, the aldol synthesis of PS should include several steps (Scheme 2): (1) binding of glycine to the PLP in the active site of the enzyme with formation of an external aldimine (EG); (2) deprotonation of the α-carbon of glycine and formation of PLP–glycine quinonoid complex (E-Quin); (3) binding of benzaldehyde and formation of the C–C bond; (4) release of PS from the active site and regeneration of the internal aldimine form of the enzyme.

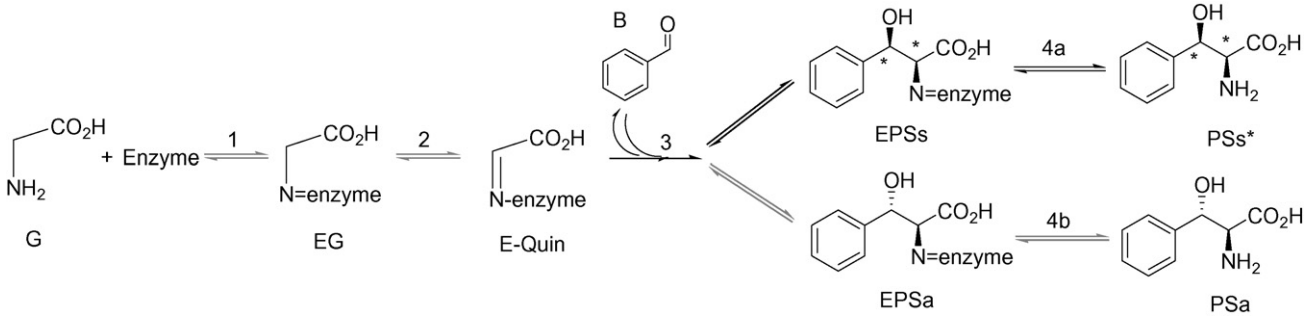
For serine hydroxymethyltransferase (SHMT) with a similar mechanism of catalysis as compared to TAs [41,42] it was shown that the equilibrium steps 1 and 4 are very rapid, and the rate limiting step could be either cleavage/formation of C–C bond or protonation/deprotonation of the glycine–enzyme complex [43,44].

The diastereomeric ratio of the product in the kinetically controlled reaction depends on the difference between the barriers for the synthesis of each isomer, while under thermodynamic control the *d.e.* depends on the thermodynamic stability of the products. On the basis of the suggested mechanism, we propose a general energy diagram for the aldol synthesis of PS, which includes the steps described above (Fig. 3).

To determine the rate-limiting step and to elucidate the relative heights of the energy barriers we investigated the distribution of a ^{13}C-label in the reaction under conditions close to equilibrium (*d.e.* ~20%, *syn*). Amounts of labeled compounds were measured by ^{1}H NMR.

Table 2
Four types of threonine aldolases in the synthesis of phenylserine under kinetic and thermodynamic control

Type	Enzyme	Kinetically controlled			Thermodynamically controlled		
		Time	*d.e.* (%)	Yield (%)	Time	*d.e.* (%)	Yield (%)
I	LTA *Pseudomonas putida*	1 min	>30(*syn*)	40	30 min	21 (*syn*)	80
	LTA *P. aeruginosa*	1 min	>30(*syn*)	10	30 min	21 (*syn*)	80
II	L*allo*TA *A. jandaei*	<1 min	*anti*	<20	5 min	27 (*syn*)	30
	L*allo*TA *Thermotoga maritima*	<1 min	*anti*	<20	5 min	20 (*syn*)	25
III	LlowTA *Saccharomyces serevisiae*	1 min	40 (*anti*)	4	5 h	22 (*syn*)	60
	LlowTA *B. bronchiseptica*	5 min	70 (*anti*)	10	n.d.	n.d.	n.d.
IV	DlowTA *Alcaligenes xylosoxidans*	1 h	85 (*syn*)	70	5 days	17 (*syn*)	80
	DlowTA *S. pomeroyi*	5 min	20 (*syn*)	10	5 days	21 (*syn*)	80



Scheme 2. NMR-studies of the ^{13}C distribution in the retro-aldol reaction catalyzed by LTA. G: glycine; EG: enzyme glycine complex; EPSs: enzyme/*syn*-PS complex; EPSa: enzyme/*anti*-PS complex; PSs*: ^{13}C-*syn*-PS; PSa: *anti*-PS. *Conditions*: 1 mL solution containing glycine (0.46 M), benzaldehyde (10 mM), PLP (50 μM), DL-^{13}C-*syn*-PS (48 mM), L/D-*anti*-PS (32 mM), TA (3 U), D_2O at 25 °C, pH 8.

The reaction mixture contained DL-*syn*-^{13}C-PS (48 mM), DL-*anti*-PS (32 mM), glycine (0.46 M), benzaldehyde (10 mM), PLP and TA (0.6 U/μmol for Thr) in compliance with Scheme 2. Reactions were carried out in deuterated phosphate buffer pH 8.0 at 25 °C in a NMR tube and the mixture composition was measured every 5 min. The assignment of labeled compounds was based on the protons bond to ^{13}C which give a doublet in the ^{1}H spectrum. The coupled signals appear as satellites of the main uncoupled signal. Thus, labeled compounds display as doublet, while unlabeled as singlet. The rate of distribution of ^{13}C between all components within the time course of the reaction gives information on the relative size of the energy barrier for the corresponding transition. Balance of labeled compounds in the reaction shows the relative mistake of the experiment.

According to the data obtained (Fig. 4), for the reaction catalysed by LTA from *P. aeruginosa* (Type I) *anti*-^{13}C-PS is initially formed 20 times faster then ^{13}C-glycine (Fig. 4A). This indicates that comparably higher activation energy is needed for the binding of glycine to the active site of TA, which makes it the rate-determining step. Thus, benzaldehyde is released during the aldol cleavage whereas glycine mainly stays at the active site and *syn*-PS can equilibrate with *anti*-PS without the release of glycine. These data might explain the reason for the low *d.e.* of PS obtained for LTA-catalyzed reaction under kinetic control. While the binding of glycine is the rate-limiting step, in the synthetic reaction the influence of the difference between energy barriers for the formation of *syn*- and *anti*-PS is of low significance ($\Delta\Delta G_{Gly} > \Delta\Delta G_{anti}$, $\Delta\Delta G_{syn}$). Due to the interconversion of diastereoisomers the more stable *syn*-PS is obtained as the major product.

Similar results were obtained for the reactions catalyzed by L*allo*TA from *A. jandaei* (Fig. 4B) and L-*low specificity* TA from *S. cerevisiae* (Fig. 4C) where the *anti*-^{13}C-PS was formed nine times faster than and twice as fast as ^{13}C-glycine, respectively.

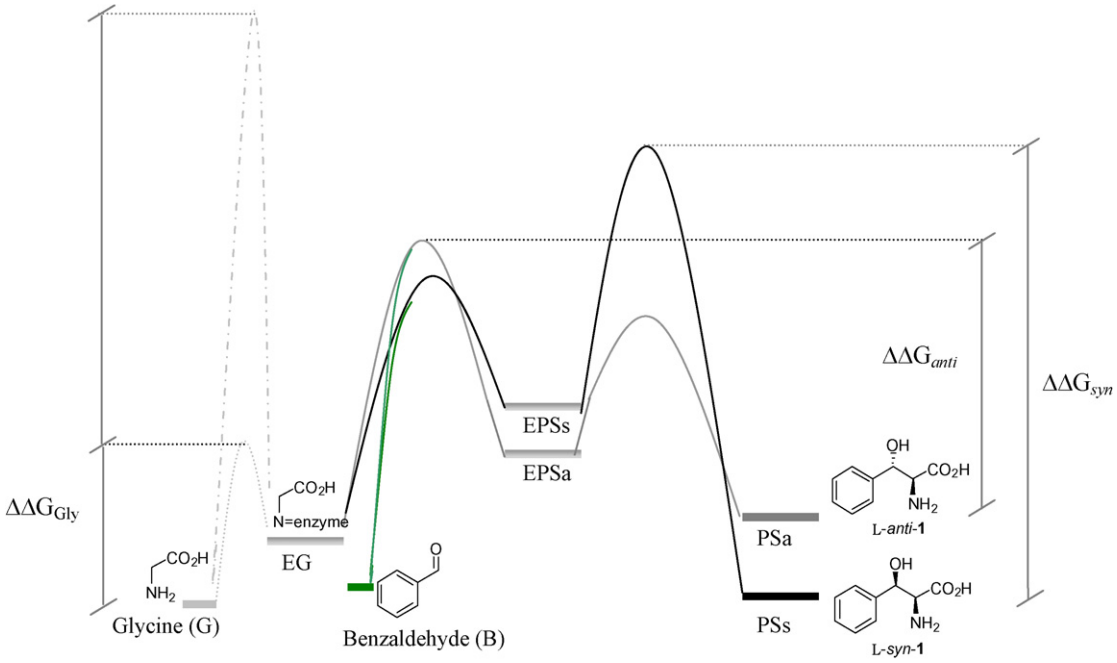


Fig. 3. Possible energy diagram for TA-catalyzed aldol and retro-aldol reactions of PS (G: glycine; EG: enzyme glycine complex; EPSs: enzyme/*syn*-PS complex; EPSa: enzyme/*anti*-PS complex; PSs: *syn*-phenylserine; PSa: *anti*-phenylserine). For $\Delta\Delta G_{Gly}$ the dotted curve (. . . .) corresponds to the DTA, the dotdashed (.-.-.-.-.) curve to the LTAs investigated.

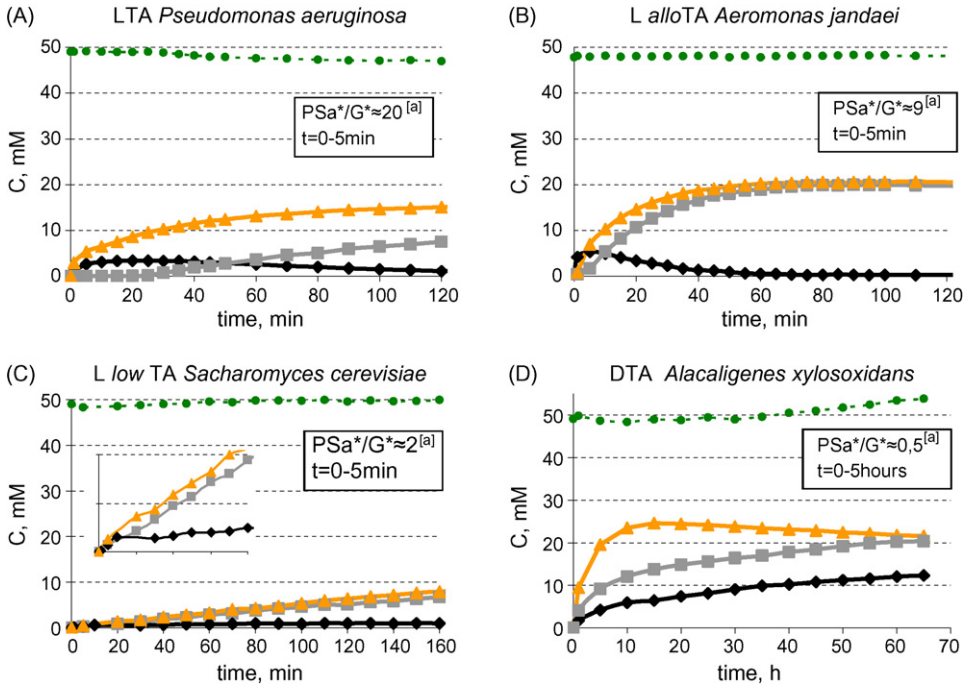


Fig. 4. Accumulation of *anti*-^{13}C-PS (♦, PSa*), ^{13}C-glycine (■, G*) and unlabeled *syn*-PS (▲, PSs) and ^{13}C balance control (●) during the aldol cleavage of DL-*syn*-^{13}C-PS catalysed by four types of TAs. *Conditions*: DL-*syn*-^{13}C-PS (48 mM), DL-*anti*-PS (32 mM), glycine (0.46 M), benzaldehyde (10 mM), PLP (50 μM) and TA. [a] PSa*/G*—ratio of the initial rates for the formation of *anti*-^{13}C-PS and ^{13}C-glycine (for LTA: $t = 5$ min; for DTA: $t = 5$ h).

A different behavior was observed for D-*low specificity* TA from *A. xylosoxidans* where direct epimerisation is very slow and, therefore, ^{13}C-glycine is the more rapidly formed product (Fig. 4D). This indicates a high difference between energy barriers for two diastereomers. According to these NMR experiments release of glycine from the active site of TA is not the rate-limiting step for this type of enzyme. Similar observations were made for D *low specificity* TA from *S. pomeroyi*.

The diastereomeric ratio of the aldol product in the thermodynamic equilibrium depends on the ΔH values of the *syn*- and *anti*-products and cannot be altered by the enzyme. However, the value of the energy barrier for each stage depends on the catalyst used and determines the rate of formation/cleavage of one or another diastereomer. For diastereoselective synthesis the different activation energies $\Delta\Delta G_{anti}$ and $\Delta\Delta G_{syn}$ are rate limiting within the reaction sequence as found for DTA catalysis. In the contrary, for the LTA investigated only low diastereoselectivities are encountered if this is not the case and the formation of the glycine/enzyme complex becomes rate determining.

4. Conclusion

In summary, four known types of threonine aldolases – LTA, L*allo*TA, L *low specificity* TA and D *low specificity* TA – were tested in the aldol and retro-aldol reactions starting from benzaldehyde/glycine or phenylserine, respectively. Their behavior in kinetically versus thermodynamically controlled reactions was compared. In the kinetically controlled reaction LTA and DTA gave *syn*-PS as the major product with moderate and high selectivity, respectively, whereas *anti*-PS was obtained for L*allo*TA and L *low specificity* TA. Under thermodynamic control a ratio of *syn*:*anti*-product of 60:40 (*d.e.* ≈20%) was obtained for all tested enzymes.

On the basis of ^{13}C distribution in the retro-aldol reaction we conclude that the rate-determining step for the L-specific TAs is the binding of glycine to the active site of the enzyme with the formation of the glycine–PLP quinonoid complex and its deprotonation. The higher the activation energy is for this step compared to the difference between the energy barriers for the formation of both diastereomers the smaller is the influence of the latter on the composition of the obtained product. This is in contrast to the observations made with DTA, where formation of a C–C bond seems to be the rate-limiting step. In this case the product composition is governed by competing the rates for the formation of *syn*- and *anti*-PS. As a consequence high selectivity (*d.e.* >95%) can be also obtained at high yields. Thus, the lowering of the activation energy barrier for the formation of the enzyme–glycine complex by means of enzyme engineering might be necessary to increase the selectivity for the LTA-catalyzed reactions and prolong the kinetic control to higher yield states. Further evaluation of the mechanism involved in the catalysis and its correlation with a crystal structure of enzymes could reveal more details of TA-catalyzed reactions.

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