Microbial aldolases as C-C bonding enzymes: Investigation of structural-functional characteristics and application for stereoselective reactions

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Abbreviations

bp	base pairs
CBB	Coomassie brilliant blue
Cbz	benzyloxycarbonyl-3-amine
Da	Dalton
DEAE	diethylaminoethyl
DERA	D-2-deoxyribose-5-phosphate aldolase
DH	dehydrogenase
DHA	dihydroxyacetone
DHAP	dihydroxyacetone phosphate
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
Ery4P	erythrose-4-phosphate
FBP	fructose-1, 6-bisphosphate
Fig	figure
FPLC	fast protein liquid chromatography
FSA	fructose-6-phosphate aldolase
Fru6P	fructose-6-phosphate
GAP	glyceraldehyde-3-phosphate
Glu6P	glucose-6-phosphate
Gly3P	glycerol-3-phosphate
HA	hydroxyacetone
HPLC	high performance liquid chromatography
IPTG	isopropyl-β-D- thiogalactopyranoside
Km	Michaelis-Menten constant
Mr	relative molecular mass
MW	molecular weight
NAD(H)	nicotinamide adenine dinucleotide
NADP(H)	nicotinamide adenine dinucleotide phosphate
NBT	nitroblue tetrazolium salt

Ni	nickel
NMR	nuclear magnetic resonance
NTA	nitrilotriacetic acid
PGI	phosphoglucose isomerase
Pi	inorganic phosphate
PCR	polymerase chain reaction
PSIG	pound per square inch gauge
Pwo	Pyrococcus woesei
RAMA	rabbit muscle aldolase
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TAL	transaldolase
Taq	Thermus aquaticus
TIM	triose-phosphate isomerase
TKT	transketolase
U	unit (= μmol/min)
UV	ultraviolet
Vmax	maximal velocity of an enzyme reaction
WT	wild type
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

(amino acids)

alanine	Ala	A	arginine	Arg	R
asparagine	Asn	N	aspartate	Asp	D
cysteine	Cys	C	glutamate	Glu	E
glutamine	Gln	Q	glycine	Gly	G
histidine	His	H	isoleucine	Ile	I
leucine	Leu	L	lysine	Lys	K
methionine	Met	M	phenylalanine	Phe	F
proline	Pro	P	serine	Ser	S
threonine	Thr	T	tryptophan	Trp	W
tyrosine	Tyr	Y	valine	Val	V

Abstract

Carbon-carbon bonding enzymes can be attractive alternatives to standard chemical methods by allowing chiral control, taking advantage of mild reaction conditions and minimizing the use of protecting groups in reactions. Fructose-6-phosphate aldolase (FSA) from *Escherichia coli* catalyses reversibly the cleavage of D-fructose-6-phosphate into dihydroxyacetone (DHA) and D-glyceraldehyde-3-phosphate (GAP). In addition, the enzyme creates new building blocks with *3S*, *4R* configuration by use of DHA as donor and various aldehydes as acceptor. The goal of this work was to investigate FSA and compare it with the transaldolase from *Bacillus subtilis* (TAL_{Bsu}) which is similar both in sequence and structure to FSA (30% identical in amino acid residues). This should help to elucidate relationships between structure and function of both enzymes, and possible applications for FSA and TAL_{Bsu} for the production of valuable sugars or sugar derivatives.

Both *fsa* and tal_{Bsu} genes were cloned with Histidine tags (6x or 10x His-tag) at the N- or C-termini to facilitate purification of the proteins. However, none of these fusion proteins (N-tagged FSA, C-tagged FSA, N-tagged TAL_{Bsu}, C-tagged TAL_{Bsu}) retained the complete enzyme activity. Both N-tagged FSA and TAL_{Bsu} did not bind to Ni-NTA column, whereas both C-tagged enzymes bound to the resin and they could be purified. Concerning quaternary structures, N- or C-tagged TAL_{Bsu} formed dimer or pentamer, while both His-tagged FSAs kept the decamer structures. This result demonstrated that His-tag would give negative influences on both FSA and TAL_{Bsu}, and it was unsuitable for assay of those enzymes. In addition, FSA had different structural stability from that of TAL_{Bsu}.

To examine the importance of several residues at the active center in FSA, three FSA mutants (Q59E, Y131A and Y131F) were prepared so as to have the corresponding amino acid residues that are present in TAL_{Bsu} and several other TALs (Gln -> Glu, Tyr -> Phe). The purified FSA Q59E protein retained approximately 66% of the wild type (WT) activity, whereas both Y131A and Y131F were completely inactive, though all three retained their decameric structures. From three-dimensional structural analysis of FSA Q59E (by the group of G. Schneider at Karolinska Institute, Stockholm), it appeared that the mutant protein's structure is identical to WT. Each mutant lost stability at high temperature and was thus denatured by heat treatment (75°C, 40min). This suggested that the Tyr131 residue has an important role for FSA activity. Indeed, a hydroxyl group at the phenyl moiety of the residue appears to be indispensable for the catalytic reaction, as the structural balance of FSA was lost by the alteration of Tyr131.

Another mutant of FSA, A129S, was assayed for its synthetic capability and compared with FSA WT. Both WT and A129S catalyzed two reactions using DHA as donor with formaldehyde or glycolaldehyde as acceptor and produced *S*-erythrulose or D-xylulose, respectively. However, A129S showed much higher activity thus yielding larger amounts of products. On the other hand, almost no difference was observed in catalytic ability between WT and A129S for a reaction using hydroxyacetone as donor. It is supposed that a hydroxyl moiety of DHA interacts with the hydroxyl group of Ser129 in FSA A129S via a hydrogen bond and changes the affinity of the enzyme towards DHA.

As further synthetic reactions, aminoaldehydes were assayed as acceptors for FSA. FSA recognized *N*-Cbz-3-aminopropanal with DHA and an aldol adduct (a precursor of fagomine) was produced even at 4 °C (performed by the group of P. Clapes in CSIC, Barcelona). This indicates a high utility of FSA in organic syntheses, the enzyme indeed could recognize large molecules including a benzene ring as acceptor and retains catalytic ability at rather low temperature.

To evolve diverse catalytic abilities of FSA and progress further application of the enzyme, random mutagenesis was adopted by use of error prone PCR technique. As a result, various mutant proteins were acquired with the alteration of 1 to 6 residues per gene. This implies that FSA mutants can be prepared with this technique and enzyme libraries could be created.

To compare FSA with TAL_{Bsu} in structure, wild-type TAL_{Bsu} was cloned in *E. coli* and purified. Three-dimensional structure analysis (by T. Sandalova in the group of G. Schneider in Karolinska Institute, Stockholm) revealed that the enzyme is a decameric protein (10 subunits of 23kDa) resulting from the dimerization of two identical pentamers. This makes TAL_{Bsu} highly similar to FSA in structure with the exception only of a shorter C-terminal helix. TAL_{Bsu} was tolerant of high temperature as FSA (at 75°C for 30-40min), and recognized different aldehydes or their phosphate derivatives as acceptors (Fru6P as donor). DHA was utilized as donor at a specific activity of about 10% of a reaction using Fru6P.

Eight chimera proteins (chimera1-8) consisting of parts of FSA and progressively truncated TAL_{Bsu} were designed and overexpressed in *E. coli* to probe structural determinants for each enzyme. However, several chimera samples (chimera1, 3, 6) showed only faint protein bands on SDS-PAGE and two (chimera2, 7) were not detected. All cell-free extracts of chimera proteins showed neither FSA nor TAL_{Bsu} activity.

Zusammenfassung

C-C-Bindungen knüpfende Enzyme können attraktive Alternativen zu normalen chemischen Methoden zur Bildung neuer chiraler Gruppen sein, damit Reaktionen unter milderen Bedingungen ablaufen können. Fruktose-6-Phosphat Aldolase (FSA) aus *Escherichia coli* katalysiert die reversible Spaltung von D-Fruktose-6-Phosphat zu Dihydroxyaceton (DHA) und D-Glycerinaldehyd-3-Phosphat (GAP). Außerdem bildet das Enzym neue Bausteine mit *3S*, *4R*-Konfiguration unter Verwendung von DHA als Donor und verschiedenen Aldehyden als Akzeptor. Das Ziel dieser Arbeit war die Untersuchung von FSA im Vergleich zur Transaldolase aus *Bacillus subtilis* (TAL_{Bsu}). Die Transaldolase besitzt eine ähnliche Sequenz und Struktur wie FSA (30% identische Aminosäurereste). Dies sollte dabei helfen, die Beziehungen zwischen Struktur und Funktion beide Enzyme aufzuklären, und mögliche Anwendungen für FSA und TAL_{Bsu} für die Produktion von hochwertigen Zuckern und Zuckerderivaten, zu untersuchen.

Die *fsa* und *tal* Gene wurden jeweils mit Histidin-Tag (6x or 10x His-tag) am N- bzw. C-Terminus kloniert, um die Reinigung der Proteine zu erleichtern. Keines dieser Fusionsproteine (FSA N-His-Tag, FSA C-His-Tag, TAL_{Bsu} N-His-Tag, TAL_{Bsu} C-His-Tag) besaß die volle Enzymaktivität. FSA N-His-Tag und TAL_{Bsu} N-His-Tag banden nicht an eine Ni-NTA-Matrix. Die beiden Enzyme mit C-Terminalem His-Tag interagierten mit der Matrix und wurden aufgereinigt. Bezüglich ihrer Quartärstruktur bildete das TAL_{Bsu} N-His-Tag oder TAL_{Bsu} C-His-Tag Dimere oder Pentamere. Hingegen behielten beide FSA His-Tag die Dekamerenstruktur bei. Dieses Ergebnis zeigte, dass der His-Tag einen negativen Einfluß auf die Enzyme ausüben kann.

Zur Untersuchung der wichtigen Aminosäuren im aktiven Zentrum von FSA wurden drei verschiedene Mutanten des Enzyms hergestellt. Dabei wurden die Aminosäurereste zu den korrespondierenden Resten des TAL_{Bsu} und den anderer Transaldolasen ausgetauscht (Gln -> Glu, Tyr -> Phe). Das gereinigte FSA Gln59Glu Protein behielt ungefähr 66% der Aktivität des Wildtyps bei, hingegen waren beide Tyr131 Mutanten (Y131A und Y131F) inaktiv. Alle drei Mutanten behielten ihre dekamere Struktur bei. Die Analyse der dreidimensionalen Struktur von FSA Gln59Glu (Messungen wurden von der Gruppe von G. Schneider am Karolinska Institut, Stockholm, durchgeführt), zeigte, dass die Struktur des Mutantenproteins identisch mit dem Wildtyp war. Alle Mutanten verloren ihre Stabilität während der Hitzefällung und denaturierten (75°C, 40min). Das Ergebnis deutet an, dass die Aminosäure Tyr131 eine wichtige Rolle für die FSA Aktivität hat. Es könnte sein, dass die Hydroxylgruppe des Phenylrests der Aminosäure für die katalytische Reaktion

notwendig ist. Eine andere Mutante, FSA A129S, wurde auf ihre Synthesefähigkeit hin untersucht und mit dem FSA Wildtyp verglichen. Beide, FSA Wildtyp und Mutante A129S, katalysieren die Reaktionen mit DHA als Donor und Formaldehyd oder Glykolaldehyd als Akzeptor. Dabei wird *S*-Erythrulose bzw. D-Xylulose gebildet. Jedoch zeigte FSA A129S höhere Aktivitäten und größere Mengen der Produkte wurden gebildet. In der Katalyseeigenschaft des Wildtyps und der Mutante A129S wurde bei Hydroxyaceton als Donor kein Unterschied wahr genommen. Es wird vermutet, dass eine Hydroxylgruppe von DHA mit der Hydroxylgruppe von Ser129 auf FSA A129S über eine Wasserstoffbrücke interagiert und so die Affinität des Mutantenenzyms zu DHA verändert.

Als Synthesereaktion von FSA wurden Aminoaldehyde als Akzeptoren eingesetzt. FSA erkannte *N*-Cbz-3-Aminopropanal mit DHA und die Aldolprodukte (Vorläufer von Fagomin) wurden sogar bei 4°C produziert (durchgeführt von der Gruppe P. Clapes in CSIC, Barcelona). Dies zeigt den hohen Nutzen von FSA in organischen Synthesen. So konnte das Enzym ein großes Molekül, einschließlich eines Benzolringes, als Akzeptor erkennen und behielt trotzdem die katalytische Fähigkeit auch bei niedriger Temperatur bei.

Zur Entwicklung erweiterten Einsatzmöglichkeiten der FSA wurden zufällige Mutationen mittels einer “error prone PCR” eingeführt. Pro FSA-Mutante fanden 1-6 Aminosäureaustausche statt. Das bedeutet, dass FSA Mutanten vorbereitet werden können und eine Gen-Bibliothek für das Enzym erstellt werden kann.

Wildtyp TAL_{Bsu} wurde in *E.coli* kloniert und gereinigt, um die Struktur mit der von FSA zu vergleichen. Die Analyse der dreidimensionalen Struktur (durchgeführt von T. Sandalova in der Gruppe von G. Schneider am Karolinska Institut, Stockholm) zeigte, dass TAL_{Bsu} eine dekameres Protein ist (10 Untereinheiten von 23kDa) und aus zwei identischen Pentameren zusammengesetzt ist. Die Struktur von TAL_{Bsu} macht die Ähnlichkeit zur Struktur von FSA deutlich mit der Ausnahme der kürzeren C-terminalen Helix. TAL_{Bsu} ist ein thermostabiles Protein ähnlich wie FSA, und erkennt verschiedene Aldehyde oder Zuckerphosphatderivate als Akzeptor (mit Fru6P als Donor). Wenn DHA als Donor verwendet wurde, zeigte TAL_{Bsu} eine spezifische Aktivität von etwa 10% bezüglich Fru6P als Donor.

Acht chimäre Proteine (Chimäre1-8) bestehend aus jeweils einem Teil der FSA Sequenz und einem Teil der TAL_{Bsu} Sequenz wurden konstruiert und die Proteine in *E.coli* exprimiert, um die Strukturdeterminanten für jedes Enzym zu untersuchen. Nur einige chimäre Proteine (Chimäre 1, 3, 6) zeigten sich als eine schwache Bande in der SDS-PAGE. Alle Rohextrakte der chimären Proteine zeigten weder eine FSA noch eine TAL_{Bsu} Aktivität.

1. Introduction

1-1. Carbon-Carbon (C-C) bond formation and aldolases

Carbon-carbon (C-C) bond formation is a key reaction in organic synthesis, because it can be used to produce highly functionalized compounds with a pair of newly generated chiral centers (Sawamura et al., 1993; Schmalz 2000). Thus, much effort has been directed to develop chemical methods that bring asymmetric C-C bond forming in synthetic chemistry (Sawamura et al., 1993; Schmalz, 2000; Seoane, 2000). With this respect, enzymes have been increasingly recognized as useful catalysts for organic synthesis (Gijsen et al., 1996; Seoane, 2000). C-C-bonding enzymes such as lyases (EC:4.1.x.x) or transferases (EC:2.2.x.x), are advantageous to add a one-, two- or three-carbon fragment onto an acceptor substrate. They can be attractive alternatives to standard chemical means by allowing chiral control, taking advantage of mild reaction conditions and minimizing the use of protecting groups in reactions (Drauz et al., 1995; Fessner, 1998; Fessner & Helaine, 2001).

Asymmetric C-C bond formation based on catalytic aldol reactions remains one of the most challenging subjects in synthetic organic chemistry, it requires auxiliary reagents in stoichiometric amounts and the use of a metal or metal-like enolate complex to achieve high stereoselectivity (Gijsen et al., 1996; Nogradi, 1995; Paterson et al., 1990; Sawamura et al., 1993; Suto et al., 2005; Trost et al., 2005). Due to the instability of such complexes in aqueous solutions, aldol reactions often should be carried out in organic solvents and at low temperature (Faber, 2004; Nogradi, 1995; Schmalz, 2000). As a consequence, the employment of aldol reactions usually requires extensive protection protocols for compounds containing polyfunctional polar groups like carbohydrates to make them lipophilic and to avoid undesired side reactions. Those requirements limit the application of conventional aldol reactions in aqueous solutions. On the other hand, enzymatic aldol reactions are performed in aqueous solutions at neutral pH without extensive protection methodology (Faber, 2004; Gijsen et al., 1996). Thus, they have attracted the interest of synthetic chemists. It is now known that aldolases operate on a wide range of substrates including carbohydrates, amino acids and hydroxy acids (Faber, 2004).

Aldolases belong to a group of lyases and they are found in most organisms. They catalyze reversible aldol reactions. For their distinct reaction mechanisms, they have

been divided into class I and class II aldolases (Schürmann Me, Dissertation 2001; Faber, 2004). Regarding the formation of the (donor) carbanion, both mechanisms are closely related to usual aldol reactions with the carbanion being stabilized via enolate- or enamine species (Fig.1-1) (Drauz et al., 1995; Faber, 2004). Class I aldolases, found in all living organisms from prokaryotes to eukaryotes including higher plants and animals, require no metal cofactor, and catalyze the aldol reaction through a Schiff-base intermediate (Fig.1-1a). The donor is covalently linked to the enzyme, involving the ε-amino group of a lysine residue to form a Schiff-base. It is followed by the formation of an enamine species with H^+ abstraction, which performs a nucleophilic attack on the carbonyl group of the aldehyde (acceptor substrate) in an asymmetric manner.

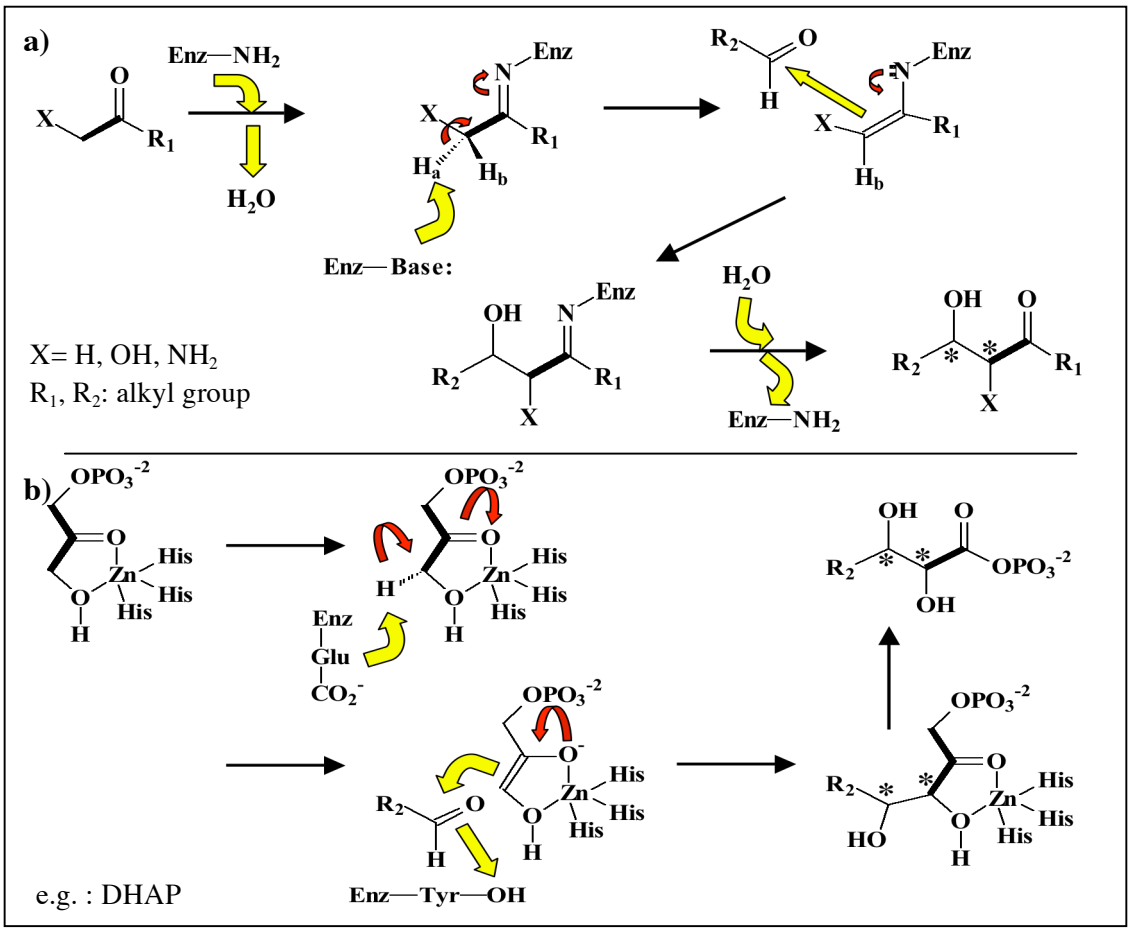
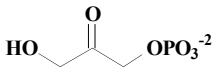
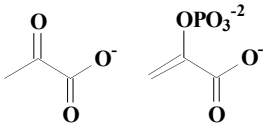
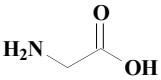
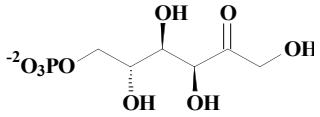


Fig.1-1. Reaction mechanisms of a) type I aldolases and b) type II aldolases; Schiff-base is formed with a lysine residue in type I, while a coordination architecture is created with a divalent cation (e.g. Zn^{2+}) in type II. Asterisks (*) indicate chiral carbon atoms whose stereochemistry depend on enzyme types (modified Faber, 2004, p274-275).

As a result, two new chiral centers are formed stereospecifically in a *threo*- or *erythro*-configuration depending on enzymes. Class II aldolases are predominantly seen in prokaryotes and lower eukaryotes such as yeast, fungi and algae. These enzymes are dependent on a divalent cation like Zn^{2+} (Drauz et al., 1995; Faber, 2004; Fessner et al., 1996). The reaction mechanism has recently been suggested to proceed through a metal-enolate (Fig.1-1b). The Zn^{2+} atom at the active site is coordinated by three N atoms of histidine residues and binds a donor substrate via the hydroxyl and carbonyl groups. This facilitates *pro*-(*R*)-proton abstraction from the donor (presumably a glutamate residue works as base) and rendering an enolate, which launches a nucleophilic attack onto an acceptor (Faber, 2004; Fessner et al., 1996).

Aldolases are conveniently classified into four groups according to their donor substrates (Table.1-1).

Table.1-1. Classification of aldolases in different donor substrates (Schoevaart, 2000; Schürmann Me, Dissertation 2001).

dependence	donor	examples
DHAP		RAMA (Fru-1,6-bis-P aldolase from rabbit muscle)
pyruvate PEP		Sialic acid aldolase NeuAc synthetase (*N*-acetylneuraminic acid)
acetaldehyde		DERA (2-deoxyribose-5P aldolase)
glycine		L-threonine aldolase
transaldolases		transaldolase B (TAL B) (from *E.coli*)

The best studied group is the one of dihydroxyacetone phosphate (DHAP) depending enzymes (Choi et al., 2001; Dalby et al., 1999; St-Jean et al., 2005), which yield a ketose-1-phosphate. Enzymes in the group are capable of forming four possible stereoisomers at C3 and C4 positions of newly built compounds (3*S*/4*R*-threo, 3*R*/4*S*-threo, 3*S*/4*S*-erythro, 3*R*/4*R*-erythro; Fig.1-2).

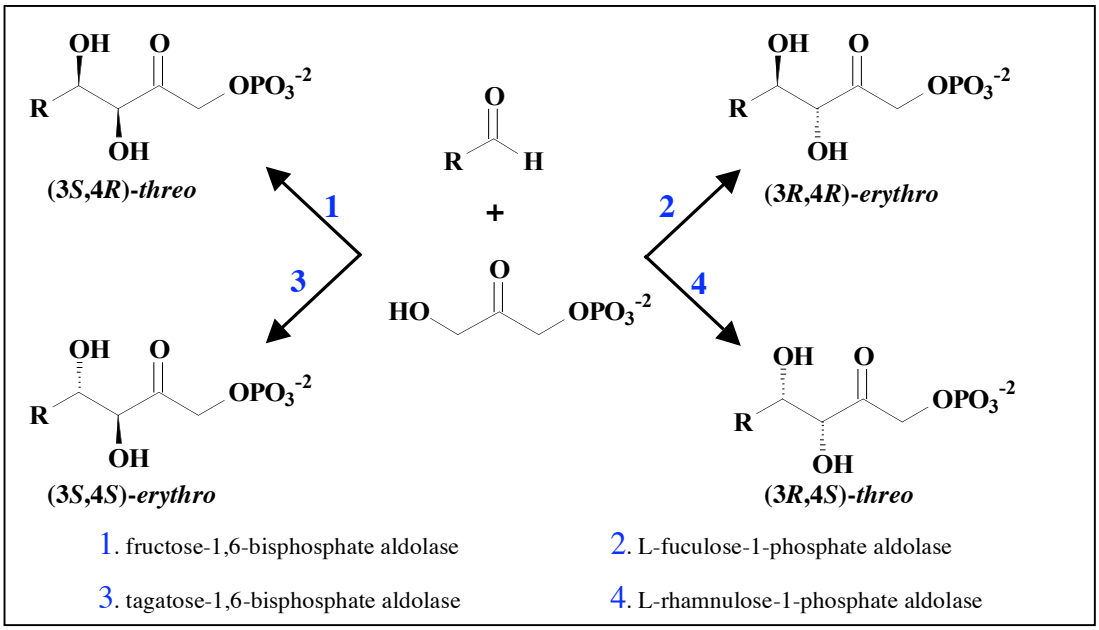


Fig.1-2. Four different stereoselectivities with dihydroxyacetone phosphate (DHAP) depending aldolases. An aldehyde as an acceptor substrate is utilized together with DHAP (Faber, 2004; Fessner, 1992; Gijsen et al., 1996).

Concerning other groups, pyruvate or phosphoenol pyruvate (PEP) depending enzymes produce 3-deoxy-2-keto acids that have a single chiral center at C4 position. Pyruvate depending aldolases have catabolic functions *in vivo*, whereas PEP depending aldolases participate in biosynthesis of keto acids. 2-deoxyribose-5-phosphate aldolase (DERA) is the only one found as aldehyde depending enzyme that accepts two aldehydes as substrates. This enzyme catalyzes the reversible cleavage of 2-deoxyribose-5-phosphate (which is the sugar moiety of DNA) into acetaldehyde and glyceraldehyde-3-phosphate (GAP). DERA yields a 2-deoxy-aldose which has an asymmetric carbon at C3 position (Schoevaart, 2000; Heine et al., 2001). Glycine depending aldolases catalyze the reversible aldol reaction of glycine with an aldehyde, and create α-amino-*β*-hydroxy

acids as products that have two new asymmetric carbons at C2 and C3. Those compounds are naturally occurring amino acids such as L-threonine or L-serine and components of complex natural products including antibiotics like vancomycin or cyclosporine (Schoevaart, 2000).

Transaldolase (TAL: EC 2.2.1.2) is a transferase in the non-oxidative branch of the pentose phosphate pathway (PPP). PPP is a metabolic pathway utilizing the 6 carbons of glucose to generate 5 carbon sugars and reducing equivalents (Sprenger, 1995; Stryer, 1988; Woodward et. al, 2002). In the pathway, NADPH as a reductant in various synthetic (anabolic) pathways like fatty acid synthesis and ribose-5-phosphate as material of nucleic acids or purine nucleotides are generated, and carbon skeletons of carbohydrates are rearranged into glycolytic/gluconeogenic intermediates by several enzymes containing TAL (Fig.1-3) (Jia, 1997; Madigan et al., 2003; Veech, 2003).

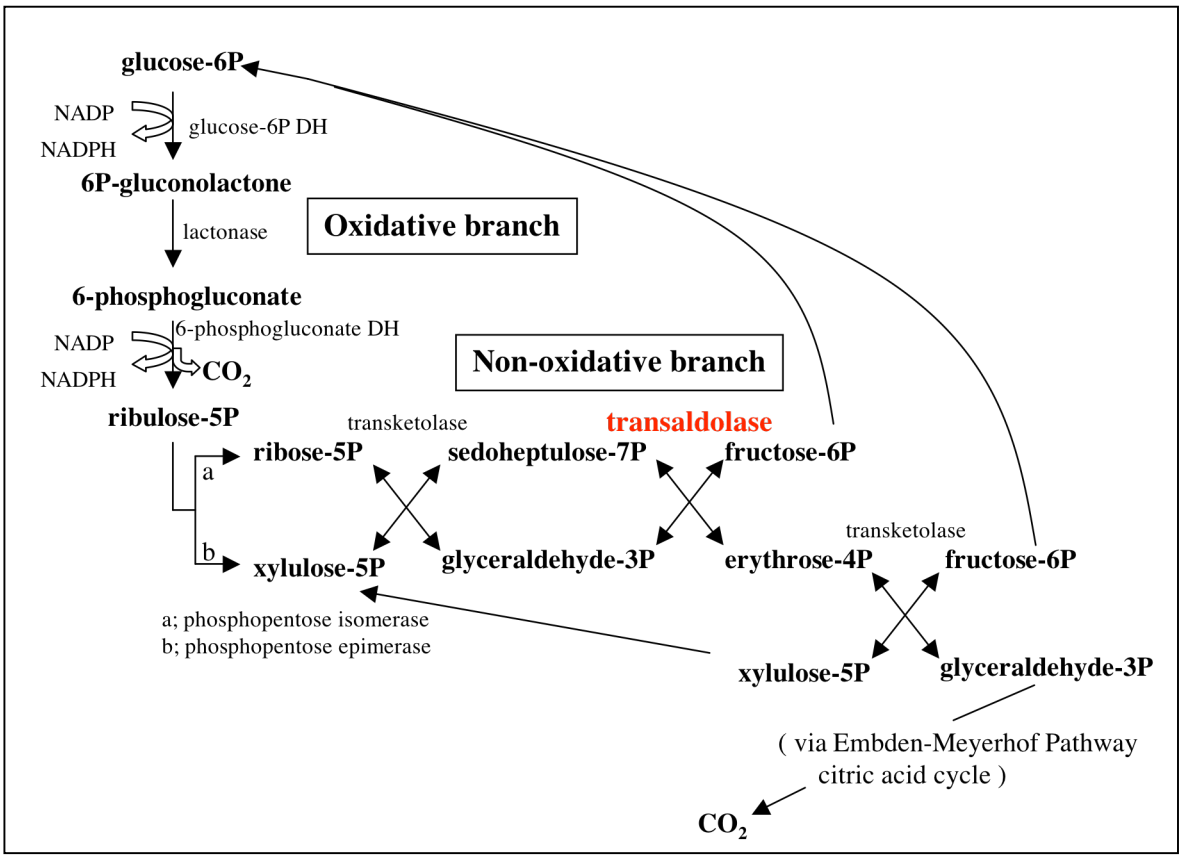


Fig.1-3. Pentose Phosphate Pathway (PPP - oxidative and non-oxidative branch) and the related enzymes. Transaldolase is depicted in red (Jia, 1997).

TAL transfers the dihydroxyacetone (DHA) moiety of a ketose (donor substrate) onto an aldehyde (acceptor substrate) and produces a ketose and an aldose that have 3*S*, 4*R* configuration. The enzyme belongs to the class I aldolase family, and occurs in a large variety of organisms such as bacteria, plants, yeast and human, briefly a ubiquitous enzyme (Horecker et,al., 1962; Jia, 1997). The physiological reaction is the reversible conversion of sedoheptulose-7-phosphate (Sed7P) and GAP into fructose-6-phosphate (Fru6P) and erythrose-4-phosphate (Ery4P) (Gonzalez-Garcia et al., 2003; Sprenger et al., 1995; Schörken et al., 2001). One half of the reaction, the Schiff-base formation and carbon-carbon bond cleavage, is similar to that by a DHAP enzyme, fructose-1,6-bisphosphate (Fru-1,6-P) aldolase. However, the reactions catalyzed by TAL and aldolase are apparently distinguished by the stability of the Schiff-base intermediates (Fig.1-4). The dissociation of the intermediate is performed in Fru-1,6-P aldolase by the release of DHAP after uptake of a proton by Schiff-base carbanion, while the intermediate in TAL is relatively stable so that the aldol acceptor can bind to active site (Horecker et al., 1961; Jia, 1997; Jia et al., 1996).

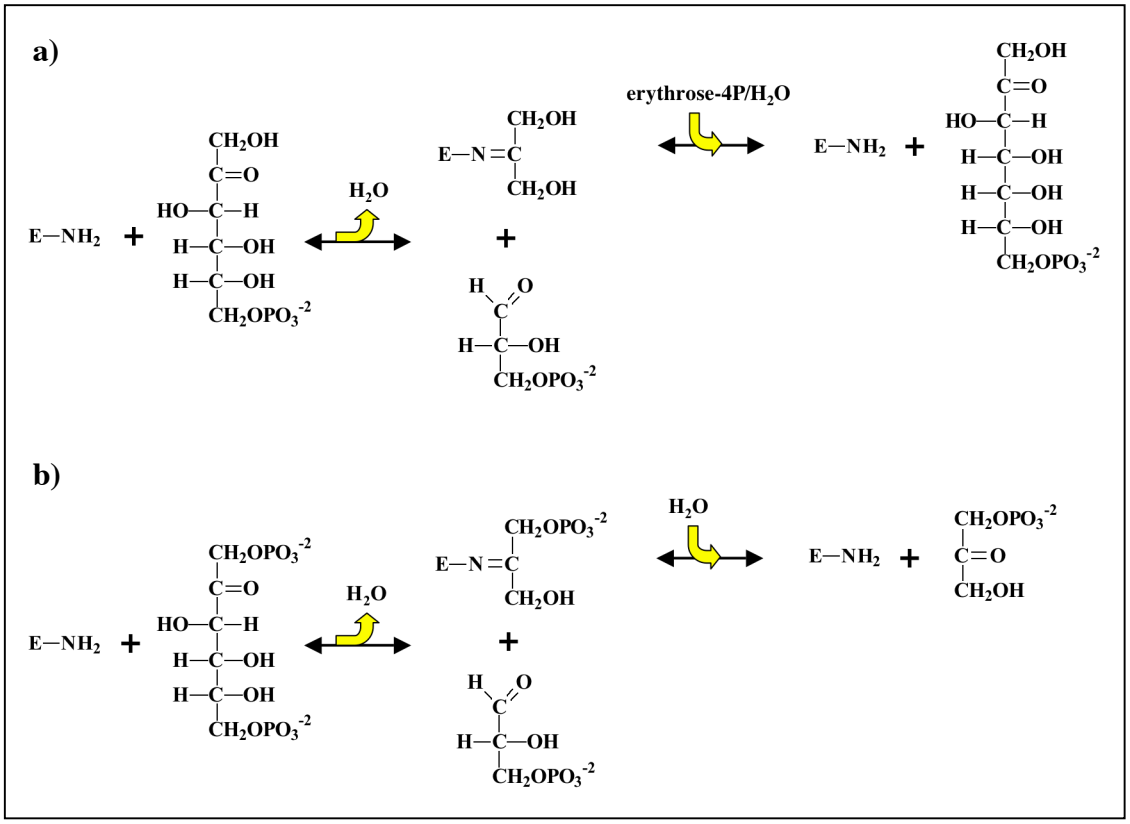


Fig.1-4. Different mechanisms of reactions catalyzed by a) transaldolase and b) Fru-1,6-P aldolase (figures adapted from Jia, 1997).

1-2. A novel aldolase, fructose-6-phosphate aldolase (FSA)

A novel TAL-related protein from *Escherichia coli* K-12, named fructose-6-phosphate aldolase (FSA), had been reported by Me. Schürmann (Dissertation, 2001). It has 50% similarity and 28% identity in amino acids to transaldolase B (TALB) from *E.coli*. The enzyme had been purified and biochemically characterized. It is a class I aldolase which does not have TAL activity but catalyzes the cleavage of Fru6P into DHA and GAP and the reverse reaction (Fig.1-5a). Such an aldol cleavage had not been reported before (Schürmann & Sprenger, 2001). In addition, this enzyme utilizes several aldehydes as acceptor (Fig.1-5b). Hydroxyacetone (HA) is also used as donor (Fig.1-5c). Therefore, various sugars or sugar derivatives with different carbon lengths can be prepared with the enzyme (Schürmann et al., 2002).

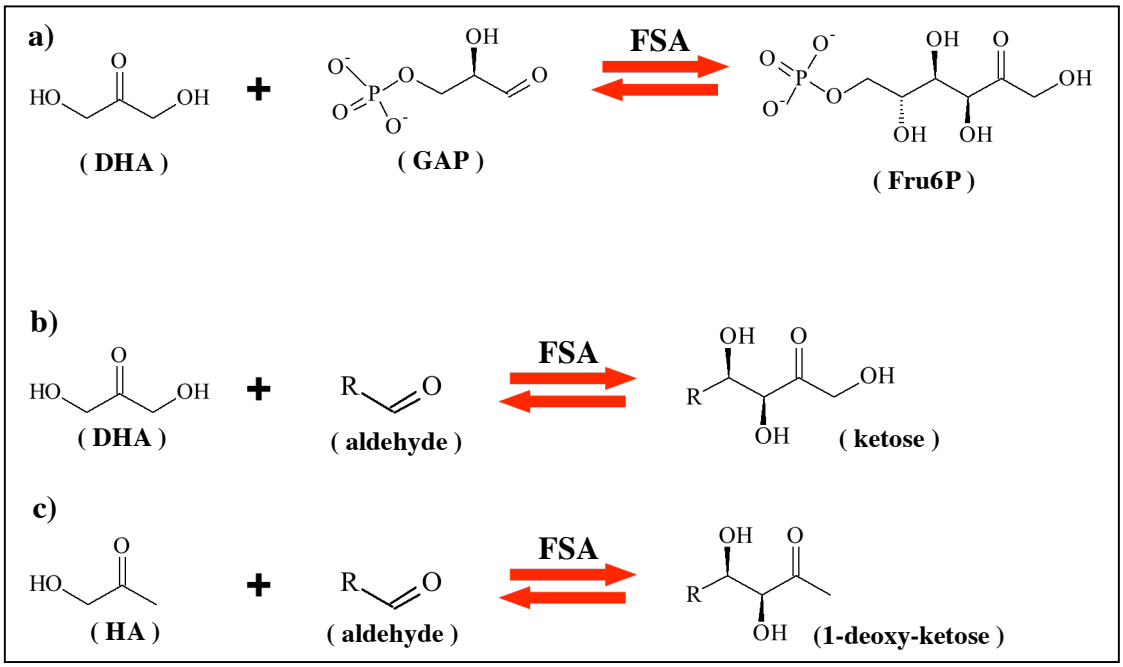


Fig.1-5. a) Forming and cleavage reaction of Fru6P with FSA, b) Reaction using aldehyde as acceptor catalyzed with FSA, c) using hydroxyacetone (HA) as donor and aldehyde as acceptor.

The molecular mass per subunit of the enzyme is 23kDa, smaller than that of TALB (35kDa, Sprenger et al., 1995). The three-dimensional structure of FSA has been determined at 1.93 A° by S. Thorell et al. at the Karolinska Institute, Stockholm,

Sweden (Thorell et al., 2002). It is a TIM -barrel protein composed of $(\beta, \alpha)_8$ barrels in the subunit and is very similar in overall structure to that of bacterial or mammalian TAL (Thorell et al., 2002). The quaternary structure of FSA is a homodecameric (230kDa) resulting from two identical pentamers forming a doughnut shape, whereas both human TAL (2x 38kDa) and TAL B (2x 35kDa) are homodimeric proteins (Fig.1-6) (Banki et al., 1994; Sprenger et al., 1995; Thorell et al., 2000; Thorell et al., 2002).

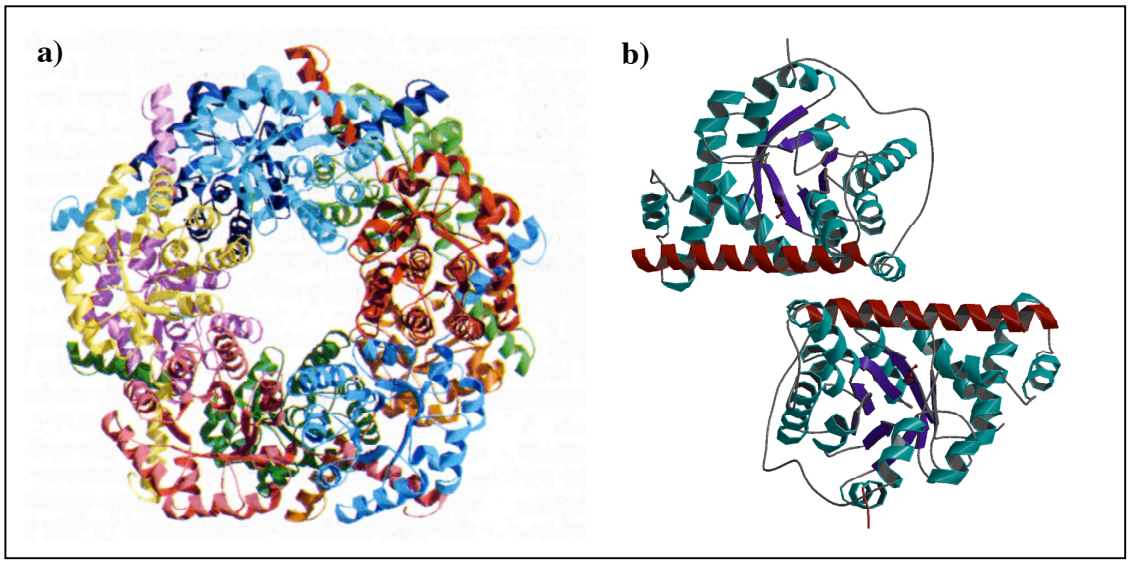


Fig.1-6. Overall structure of a) FSA and b) TAL B from *E.coli*. FSA consists of two identical pentamers and has a doughnut-like shape, while TAL B is a homodimeric protein. C-terminal helixes are characteristically different between FSA and TAL B (Thorell et al., 2002).

The distinctive structure of FSA is the interaction of subunits within a pentamer, the C-terminal helix from one monomer runs across the active site of the neighboring subunit and shields the site partially (Fig.1-7b). It is quite different from those of "classical" TALs from *E.coli* (TAL B), mouse and human (molecular mass of the subunit; 35-40 kDa - dimer formation), the helices in those TALs fold back and cover the active site of the identical subunit (Fig.1-6b). Such an interaction can make FSA more compact and physically stable than classical TALs and create it as a unique homodecameric protein, although it is similar to classical TALs in monomer subunit structure.

Data bank researches with total genome sequences from various eu- and archaebacterial microorganisms by Me. Schürmann & G. A Sprenger revealed FSA homologous proteins, "small transaldolases" (in a size range of 23-24kDa, approximately 220 amino acids), some of them were experimentally proven to be transaldolases (Schürmann Me, Dissertation 2001; Schürmann & Sprenger, 2001).

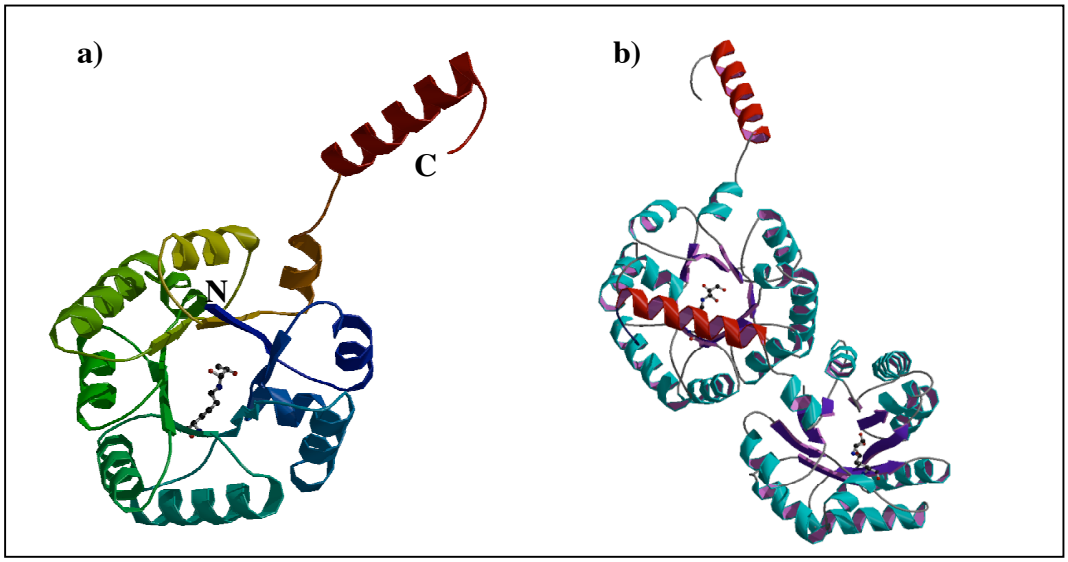


Fig.1-7. a) structure of FSA monomer; N: N-terminal (blue ribbon), C: C-terminal (red ribbon), b) interaction of one monomer with a neighbor subunit in a part of pentamer structure; C-terminal helix of a monomer subunit runs in the proximity of the active center of the neighboring subunit and covers the site like a lid (Thorell et al., 2002).

Among those proteins, "transaldolase C (TAL C)" is strikingly similar to FSA (identity: 70%) in amino acids and had been lacking TAL activity. It, indeed, had shown FSA activity although at lower specific activity when compared with FSA. Other small TALs, from *Bacillus subtilis* (identity: 30%) or an extreme thermophilic eubacterium, *Thermotoga maritima*, (identity: 30%) had encoded TAL activity but no FSA activity (Schürmann & Sprenger, 2001). Another small TAL from an archaeon, *Methanocaldococcus jannaschii*, also has a similarity to FSA (identity: 32%) and had been investigated by Soderberg et al., (2004). Those three TALs commonly have partial identities (ca. 30%) to FSA in amino acid sequence and encode TAL activity lacking FSA activity. However, they are highly temperature stable (not denatured at more than

50°C) like FSA. Another type of class I aldolases, fructose-1,6-bisphosphate aldolase (FBPA- DHAP depending aldolase) from an archaeal organism, *Thermoproteus tenax*, had been investigated and compared with FSA recently (Lorentzen et al., 2003a, 2003b, 2005; Siebers et al., 2001). Ninety-nine of 220 residues of FSA monomer had been found to be structurally equivalent to the FBPA monomer (263 residues). The sequence identity had resulted in 15% for the structurally equivalent residues, β4 of FSA corresponds to β6 of the FBPA but each enzyme had not utilized the substrate of the other's (Lorentzen et al., 2003a). In structure, both look similar and consist of two identical pentamers, however, the C-terminal helix of the FBPA does not extend into the neighboring subunit (Fig.1-8), and complete different positions of FSA and FBPA pentamers are observed. FSA decamer is assembled with the C-termini of the TIM barrels facing each other, whereas FBPA has the N-terminal sides facing each other (Lorentzen et al., 2003a). Those studies have shown that FSA is more closely related to small TALs, although FBPA and FSA catalyze quite similar reactions.

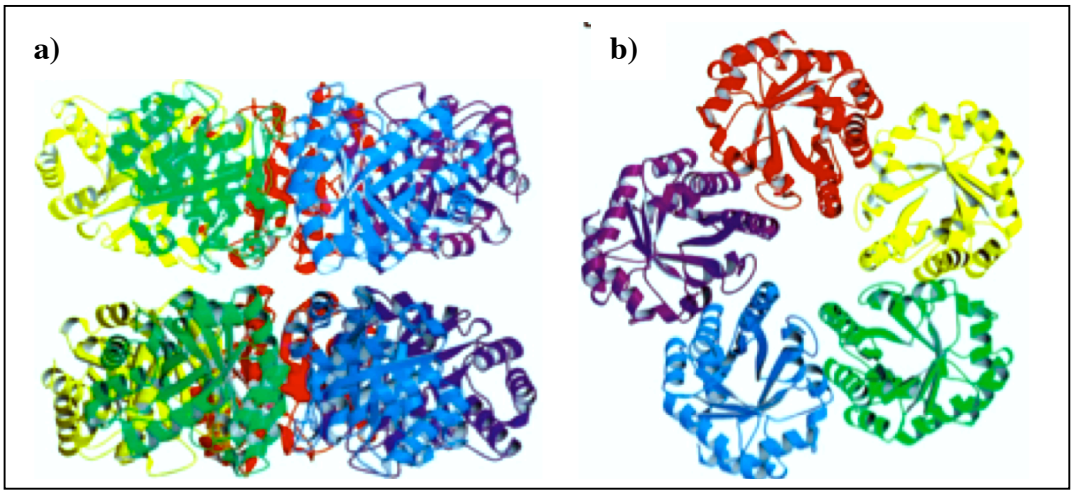


Fig.1-8. a) Side-view of crystal structure of FBPA from *T. tenax*, b) Overview of a pentamer of the FBPA, it resembles FSA pentamer structure but the C-terminal helix of FBPA does not penetrate the neighboring subunit. (cited from Lorentzen et al., 2003a).

M. Schürmann had reported that small TAL from *B. subtilis* (TAL_{Bsu}) had a decameric or dodecameric structure and that DHA could be accepted as a donor (Schürmann & Sprenger, 2001). A sequence alignment of FSA and TAL_{Bsu} shows high similarity, both enzymes have an almost identical secondary structure (Fig.1-9) (Schürmann & Sprenger, 2001; Thorell et al., 2002). It implies that FSA and small TALs share a common

evolutionary origin. The true physiological function of FSA isn't yet clear and it is speculated that the enzyme may be correlated with pentose phosphate pathway or glycolysis system (Schürmann Me, 2001).

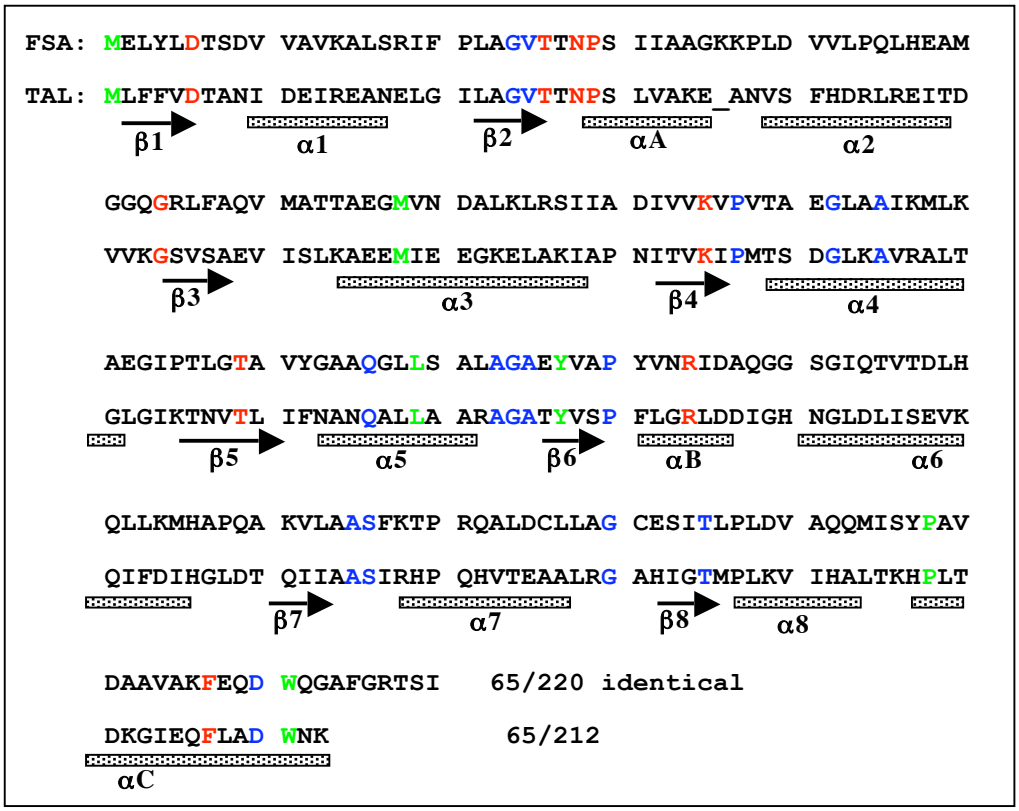


Fig.1-9. Comparison of FSA with TAL_{Bsu} in amino acid sequence with secondary structure indicated as α or β; red: invariant residues in whole TAL family, blue: invariant residues in FSA/small TALs family, green: > 90% conserved in FSA/small TALs family (Thorell et al., 2002).

1-3. Production of sugars and sugar derivatives using aldolases

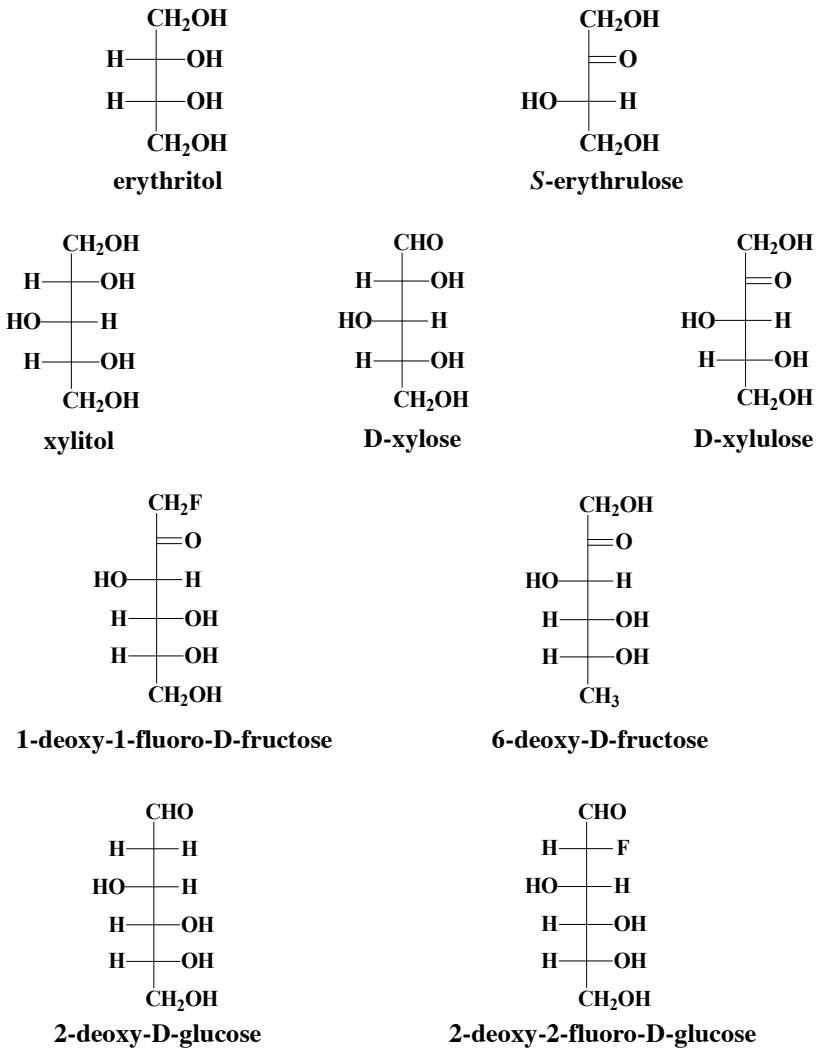
In biological production of useful sugars or the derivatives, many reports have been published (Ahmed, 2001a, 2001b; Leang et al., 2003; Moonmangmee et al., 2002). However, most of them are descriptions of the utilization of redox reactions or isomerization by biocatalysts including whole cells or fermentative production (Table.1-2) (Ahmed, 2001a). Those methods leave still challenging tasks in the high cost, the reaction efficiency, the by-products and the limitation of application for different products preparation. As mentioned, FSA can accept several aldehydes with DHA or HA and produce new keto-sugars, the phosphate derivatives and

1-deoxy-sugars. In a similar fashion, TAL_{Bsu} can recognize several aldehydes as acceptor substrates with Fru6P or DHA (Schürmann Me, 2001). Both enzymes yield a ketose which has *3S,4R* configuration in the stereospecificity with various lengths of the carbon chain from C4 to longer C backbones such as erythrulose, xylulose, deoxysugars or else, which is dependent on acceptor substrates.

Table.1-2. Examples of biological simple sugar productions.

product	starting material / organism	method / catalyst	reference
S-erythrulose	erythritol	oxidation	Moonmangmee et al.
	Gluconobacter frateurii	whole cells	2002
D-xylulose	D-arabitol	oxidation	Moses&Ferrier.
	Acetobacter suboxydans	whole cells	1962
	D-xylose	isomerization	Chiang et al.
	Fusarium oxysporum lini	whole cells	1981
	xylitol	oxidation	Doten et al.
	Providencia stuartii etc.	whole cells	1985c
D-arabitol	D-glucose		Ahmed
	Candida famata	whole cells	2001b
	D-glucose		Povelainen et al.
	*Bacilus subtilis**	whole cells	2006
D-tagatose	dulcitol	oxidation	Izumori et al.
	Arthrobacter globiformis	whole cells	1984
L-erythrose	*S*-erythrulose	isomerization	Mizanur et al.
	*Acinetobacte*r sp.	whole cells	2001
L-xylulose	xylitol	oxidation	Doten et al.
	*Erwinia uredovora**	whole cells	1985a,b
L-ribulose	ribitol	oxidation	Adachi et al.
	Gluconobacter suboxydans	whole cells	2001
L-sorbose	D-sorbitol	oxidation	De Wulf et al.
	Gluconobacter oxydans	whole cells	2000
L-glucose	L-fructose	isomerization	Leang et al.
	Klebsiella pneumoniae	isomerase	2003

*; Genetically modified strains were utilized



C4: erythritol (non-calorie sugar)

S-erythrulose (self-tanning compound in sunscreen or cosmetics)

C5: xylitol (low-calorie sugar, anti-cariogenic function)

D-xylose (activation of bacteria in intestine, antifungal compound)

D-xylulose (used in the production of xylitol or xylose etc.)

C6: 1-deoxy-1-fluoro-D-fructose (component of a tracer for sugar metabolism)

6-deoxy-D-fructose (used in flavors)

2-deoxy-D-glucose (anti-metabolite of glucose with antiviral activity)

2-deoxy-2-fluoro-D-glucose

(biochemical marker for positron emission tomography (PET))

Fig.1-10. Examples of structures and utilities of several sugars and their derivatives in industry

Some of those sugars or the derivatives are valuable in industry (e.g. Fig.1-10) and can be the initial materials or precursors for the preparation of functional foods, antimicrobial drugs, cosmetics and so on (Ahmed, 2001a). In this respect, FSA and TAL_{Bsu} can be more attractive and applicable novel tools due to the production of various useful sugars by taking advantage of an inexpensive donor compound like DHA. In addition, both might accept several amino aldehydes and create new building blocks, nitrogen-containing sugars such as amino- or imino-sugars. Those compounds would be important medical agents or the precursors for antimicrobial, antitumor and antidiabetic drugs (Asano et al., 1994; Asano et al., 1998; Asano, 2003; Kwon et al., 2005; Lu et al., 2003; Takahata et al., 2003), although amino aldehydes are labile and the commercial products are not available. Amino aldehydes should be prepared by adding protecting groups, which may interfere with enzymatic activity. A Spanish group (P. Clapes, CSIC, Barcelona), collaborating with our group, had applied aminoaldehydes with DHAP as donor to RAMA and obtained aldol adducts (Espelt et al., 2003) (e.g. Fig.1-11). Von der Osten et al. (1989) had reported the utilization of DHAP aldolase from *E.coli* (type II aldolase) with 3-azido-2-hydroxypropanal as acceptor and DHAP or DHA as donor to prepare for iminosugars. This indicates the potential that FSA or TAL_{Bsu} would catalyze reactions using amino aldehydes and nitrogen-containing sugars could be produced.

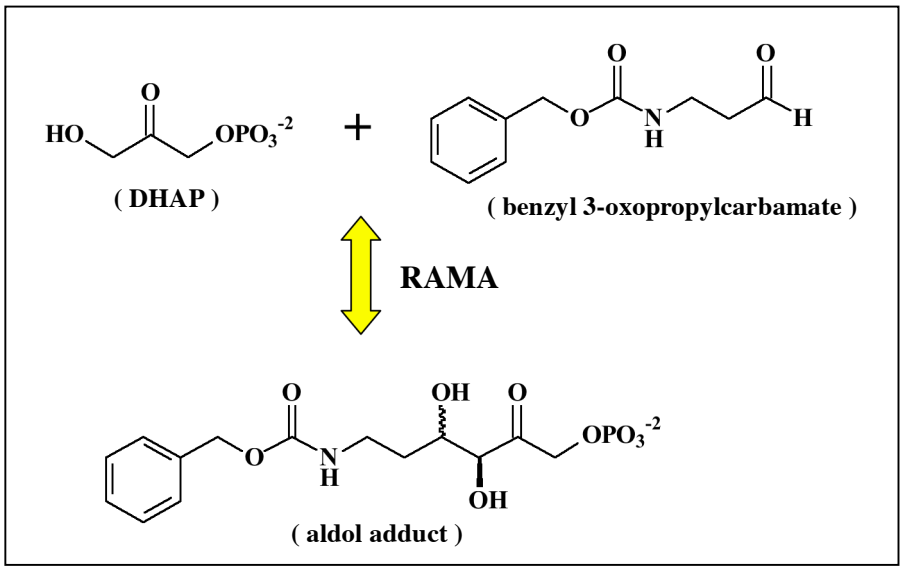


Fig.1-11. An example of aldol reaction using an amino aldehyde catalyzed with DHAP aldolase by a group of P. Clapes (Espelt et al., 2003).

This study was the successive work of a theme, "Structural-functional characterization for bacterial aldolases and transaldolases" in a collaborative research center (RWTH Aachen/ Research center Jülich; SFB380). Targets of the work was further elucidation of structural-functional characteristics of two similar aldolases, FSA and small TAL_{Bsu}. In addition, it was an important task to make up enzymatic systems for producing useful sugars or the derivatives with the aldolases. In previous work, biochemical characters of both enzymes had been investigated and the potential of FSA or TAL_{Bsu} as useful biocatalyst had been shown (Schürmann, 2001). Thus, main goals of this dissertation were the followings:

1. Investigation of important residues at the active center in FSA through preparation of mutant proteins

2. Comparison of FSA with TAL_{Bsu} in structure and function by analysis of 3-dimensional protein structures and mutant enzymes

3. Evaluation of the production of valuable keto-sugars such as *S*-erythrulose, D-xylulose or 1-deoxysugars with the enzymes or their mutants

4. Assessment of an aminoaldehyde as a novel acceptor for FSA or TAL_{Bsu} (in cooperation with the group of P. Clapes, CSIC, Barcelona)