4. Discussion

4-1. Characteristics of FSA wild type and its mutants in structure and function

Considering the structure of FSA monomer, an N-terminal His-tag might be buried internal of the molecule and the tag could not access nickel ions (Ni) on the resin in the column, while C-terminal His-tag might be located close to the surface of FSA and bound to Ni. Interestingly, both His-tagged enzymes did not retain complete activities, although their quaternary structures were held. Especially the C-His-tagged protein kept only 10% of the activity of wild type (WT) (N-His-tagged had 50% of WT's), 6 consecutive histidine residues at C-terminal could be a larger disturbance for substrates to approach the active centers of FSA. Otherwise, both His-tags may give distortion and the forms of active centers are contorted.

Preparation of FSA WT under 3 different conditions resulted in finding the use of pJF119EH vector for gene expression and two columns (Q-Sepharose, hydroxyapatite) for purification of protein (condition-2). In that way, FSA was obtained effectively with higher specific activity than under other conditions. Expression with pJF119EH vector brought lower purification factors with high amounts of protein quantity. It implies that larger quantity of FSA was initially contained in the cells (DH5α pJF119fsa) than in BL21(DE3)Star pLysS pET16. The sample using hydroxyapatite column as first one (condition-3) showed lower specific activity than sample in condition-2, though both cultural conditions were identical. It appeared that a phosphate buffer gave negative influences to FSA and it became slightly inactive during long exposure in the buffer. Me. Schürmann reported that phosphate binding sites at the active center had high affinity to phosphate groups and inorganic phosphate also could be inhibitors to FSA by the occupation of binding sites (Schürmann Me, 2001).

Two other vectors (pUC or pET) were used to prepare mutant enzymes before pJF 119EH vector was examined. Earlier, FSA WT had been purified and crystallized by making use of a pUC vector (Schürmann Me, 2001). Thus, it was practical to utilize a pUC vector for the expression of mutant proteins so as to compare FSA mutant proteins with FSA WT in three-dimensional structure. Three mutants, Q59E, Y131A and Y131F had unstable structures and were denatured by heat treatment (75°C for 40min), although they retained decamer structures. Since single amino acid alteration brought such instability, FSA appears to be a sophisticatedly designed protein by nature.

Particularly, those amino acid residues can be crucial to keep the stability or their original structural forming. Those play important roles in function, Tyr131 can hold and stabilize a water molecule which participates in a proton relay (Thorell et al., 2002). Mechanistic studies in many type I aldolases have suggested that the side-chain hydroxyl group of tyrosine residue C-terminus might work as the proton-abstracting general base (Heine et al., 2001), but FSA has relatively short C-terminal loops and there is no corresponding Tyr residue at C-terminus which could act as a flexible lid as seen in many other α/β barrel enzymes (Thorell et al., 2002). Thus, Tyr131 of FSA may have such function because of being at the active center. It was considered that FSA Y131A or Y131F should be purified and crystallized to solve the question. In case that the water molecule would exist at the ordinary position in those mutants, the hydroxyl group of Tyr131 may have not only a role as holder of the water molecule but also other functions for the reaction. Both mutant enzymes, Y131A and Y131F, however, showed an almost complete loss in activity. Gln59 could take part in the transfer of a proton directly with an amido group of the side chain. Even if Gln59 is exchanged into Glu, a carboxyl moiety of the glutamate can work instead of the amido moiety of Gln. As judged from enzyme activity in crude extract, the mutant protein kept 97% of the WT activity. But the purified enzyme retained only 66% of purified WT's in the activity. Many steps in purification including the exchange of buffers and concentration of the protein solution repeatedly or a buffer (e.g. $(NH_4)_2SO_4$ for Phenyl Sepharose F.F. column) could give the negative influence for the activity of the mutant protein.

4-2. Synthetic potential of FSA

FSA or the mutant A129S had been shown to have high potential as catalysts to produce valuable substances such as *S*-erythrulose, D-xylulose or a 1-deoxy-D-xylulose with high stereoselectivity. Up to now, there is no report that *S*-erythrulose production was won from simple and inexpensive compounds such as DHA and formaldehyde except the reaction using Fru6P and formaldehyde with TalB (0.4 U/mg: Schörken, 1997). A group had found several thermotolerant *Gluconobacter* species that oxidize *meso*-erythritol into *S*-erythrulose (Adachi et al., 2003; Moonmangmee et al., 2002), however, it was an oxidative reaction of a C4 carbon material, no carbon chain was built up newly. In addition, it seems to be difficult to obtain large advantage economically by make use of *meso*-erythritol as the initial substance (Table.4-1). Kochetov et al. had

reported *S*-erythrulose production from D-xylulose-5P in one-substrate reaction or from two molecules of glycolaldehyde by use of *Saccharomyces cerevisiae* transketolase (Bykova et al., 2001; Sevostyanova et al., 2004; Solov'eva et al., 2001). However, there is not large advantage in cost, compared with the synthesis from DHA and formaldehyde (Table.4-1).

Table.4-1. Commercial prices of chemical compounds related to enzyme reactions

reagent	purity	quantity	€*[1]/unit	supplier	EUR/mol
DHA (dimer	-97%)	500g	90.40	Sigma	17
hydroxyacetone	(> 90%)	500mL	43.50	Fluka	7
formaldehyde	(38%)	500mL	49.50	Sigma	7
glycolaldehyde (dimer)		5g	74.00	Sigma	907
S-erythrulose	(60%)	250mg	99.00	Sigma	91,159
D-xylulose	(95%)	250mg	720.00	Sigma	455,131
1-deoxy-D-xylulose		1g	1500.00*[2]	Omicron Bio.	201,195*[2]
DHAP (dilithium salt	-95%)	100mg	347.00	Fluka	664,487
β-hydroxypyruvate	(>97%)	1g	116.90	Fluka	14,734
meso-erythritol	(>99%)	100g	145.50	Fluka	179
D-arabitol	(>99%)	250g	1168.20	Sigma	718
xylitol	(>98%)	5kg	678.00	Fluka	21

*1; Prices are excerpted from supplier catalogs of booklet 2006-2007 or online 2006

*2; Currency is US $

Other reports were the reaction from *β*-HP (hydroxypyruvate)+glycolaldehyde with transketolase as shown by Bongs et al., (1997). Thus, *S*-erythrulose production from DHA and formaldehyde could bring high advantage due to low material cost (the commercial product is approximately 3800 times as much in price as the amount of both initial materials (Table.4-1)). Concerning D-xylulose production, microbial or enzymatical methods were reported (Ahmed, 2001a; Chiang et al., 1981; Doten & Mortlock 1985a, 1985b, 1985c; Moses et al., 1962), but those were applications of intramolecular conversions such as the oxidation of D-arabitol into D-xylulose or

isomerization from xylose. In the reactions, whole cells were used as catalysts. Those fermentative methods appeared to be difficult to find and control the optimal condition for D-xylulose production (Chiang et al., 1981) or to require using mutant strains for the effective production (Doten & Mortlock, 1985a, 1985b, 1985c), which would be unfavorable in industry. Utilization of FSA has advantages with respect to taking a simple reaction condition and a cell free system.

Specific activities of 3 reactions are shown on table.4-2. Values with WT were rather low, compared with the data by Schürmann - 0.09 U/mg (DHA+glycolaldehyde) and 8 U/mg (HA+glycolaldehyde), respectively, though assay conditions were different from each other (Schürmann Me, 2001) (the detail of assay condition and a reaction of DHA+HCHO were not mentioned). It implied that 3 reactions were done with qualitative assay and the conditions to measure enzyme activities were far from optimal. In fact, excess proteins were added in reaction mixtures (0.4~1.0 mg/mL) and samples were assayed only after several hours to make it possible to detect products with HPLC. Virtually, a reaction, HA+glycolaldehyde, gave approximately 3 times higher specific activities (in decreasing of HA) at 1h of incubation time (0.40 U/mg with WT or 0.48 U/mg with A129S). It demonstrates that higher specific activities may be expected by optimization of reaction conditions including optimized protein quantities.

Table.4-2. Specific activities of reactions with FSA WT or FSA A129S

	WT		**A129S**		**reported*[1]**
	specific act. *[5] (U/mg)	protein conc. (mg/mL)	specific act. *[5] (U/mg)	protein conc. (mg/mL)	specific act. (U/mg)
DHA+HCHO *[2]	0.09	0.94	0.15	0.94	no data
DHA+glycolaldehyde*[3]	0.01	0.94	0.21	0.94	0.09
HA+glycolaldehyde*[4]	0.14	0.41	0.14	0.43	8

*1; Values are taken from the dissertation of Schürmann (Schürmann Me, 2001)
The assay condition was not same as that in this work

*2; DHA:100mM, HCHO:200mM, measurement of *S*-erythrulose concentration

*3; DHA:200mM, glycolaldehyde:130mM, measurement of D-xylulose concentration

*4; HA:50mM, glycolaldehyde:200mM, measurement of residual HA concentration

*5; Specific activities are averaged for 6 or 8 hours: DHA+formaldehyde (after 8h), DHA+glycolaldehyde (after 8h) and HA+glycolaldehyde (after 6h)

4-2-1. Hydroxyacetone and further donors

As reported by Me.Schürmann, HA showed higher activities than DHA with some acceptor substrates except GAP (Schürmann Me, 2001). In fact, FSA WT appeared to catalyze the reaction using HA and glycolaldehyde (1-deoxy-D-xylulose-like compound was detected with HPLC). It indicates that FSA would produce some interesting 1-deoxysugars in combination with different aldehydes by using HA as a donor. To investigate donor substrate specificity of FSA further, *β*-hydroxypyruvate (CH_2OH-CO-COOH) and *β*-fluoropyruvate (CH_2F-CO-COOH) were examined with formaldehyde. However, they were no substrate for FSA and no reaction product was found (data not shown). A carboxyl group of those compounds may be too expanded and it can be a hindrance for the substances to access the active center. If fluoro-compounds such as 1-fluoro-3-hydroxyacetone (CH_2F-CO-CH_2OH), 1-fluoro-1-hydroxyacetone (CH_3-CO-CHFOH) or 1-fluoro-dihydroxyacetone (CHFOH-CO-CH_2OH) would be prepared and utilized as a donor for FSA, various and unique fluoro-compounds could be produced. They would be of interest for medical application for PET.

4-2-2. Application of aminoaldehydes to FSA reaction

Regarding the application of acceptor substrate, FSA recognized an aminoaldehyde as an acceptor substrate and a new product was formed at 25°C or even at 4°C (demonstrated by J. A Castillo (CSIC, Barcelona)). The conversion rate was at least 60% at 24h in the reaction at 4°C and the stereoisomeric purity was more than 99%. Specific activities were lower than 0.08 U/mg (initial conc. of aminoaldehyde; 200mM, analysis at 3h), however, it could be improved by change of reactive conditions such as higher reaction temperature or using well-purified protein with optimized quantity.

Fagomine was prepared from the aldol adduct. It has inhibitory effects on glucosidases or hepatic glycogen phosphorylase. Fagomine can be an antihyperglycemic agent for the treatment of diabetes (Asano, 2003; Asano et al., 1994, 1998; Takahata et al., 2003).

Four other aminoaldehydes were probed at 25°C by J. Castillo. Only *N*-Cbz-glycinal (*N*-(benzyloxycarbonyl)-2-aminoethanol) was accepted among those aminoaldehydes, whereas (*S*)-*N*-(benzyloxycarbonyl)-alaninal ((*S*)-*N*-Cbz -alaninal), (*S*)-*N*-Cbz-prolinal and (*S*)-*N*-Cbz-valinal) were not used. The product was detected with HPLC in the reaction using *N*-Cbz-glycinal as acceptor, while no product was observed in reactions

with other three aminoaldehydes. It appeared that three aminoaldehydes could not to be accepted by FSA. Those might be too large molecules as acceptor substrates and could not be adopted, though various aldehydes with different length of the carbon chain (acetaldehyde, propanal, butanal, pentanal and hexanal) seemed to work as acceptor substrates for FSA and new peaks corresponding to products were detected with HPLC (performed by J. Castillo: data not shown).

In the conventional method at the group of Clapes, DHAP had been utilized as donor for RAMA and the aldol reaction was done with Cbz-3-aminopropanal (Espelt et al., 2003). DHAP had been purchased or chemically prepared from DHA dimer with 5 steps. A product from DHAP and an aldehyde by RAMA should be dephosphorylated at the final step (Fig.4-1). As mentioned in introduction, there are difficulties for the utilization of DHAP due to its stability and cost. The preparation of DHAP from DHA is troublesome work. Thus, it can be of advantage in preparation of aldol adducts to use DHA as an initial material with FSA.

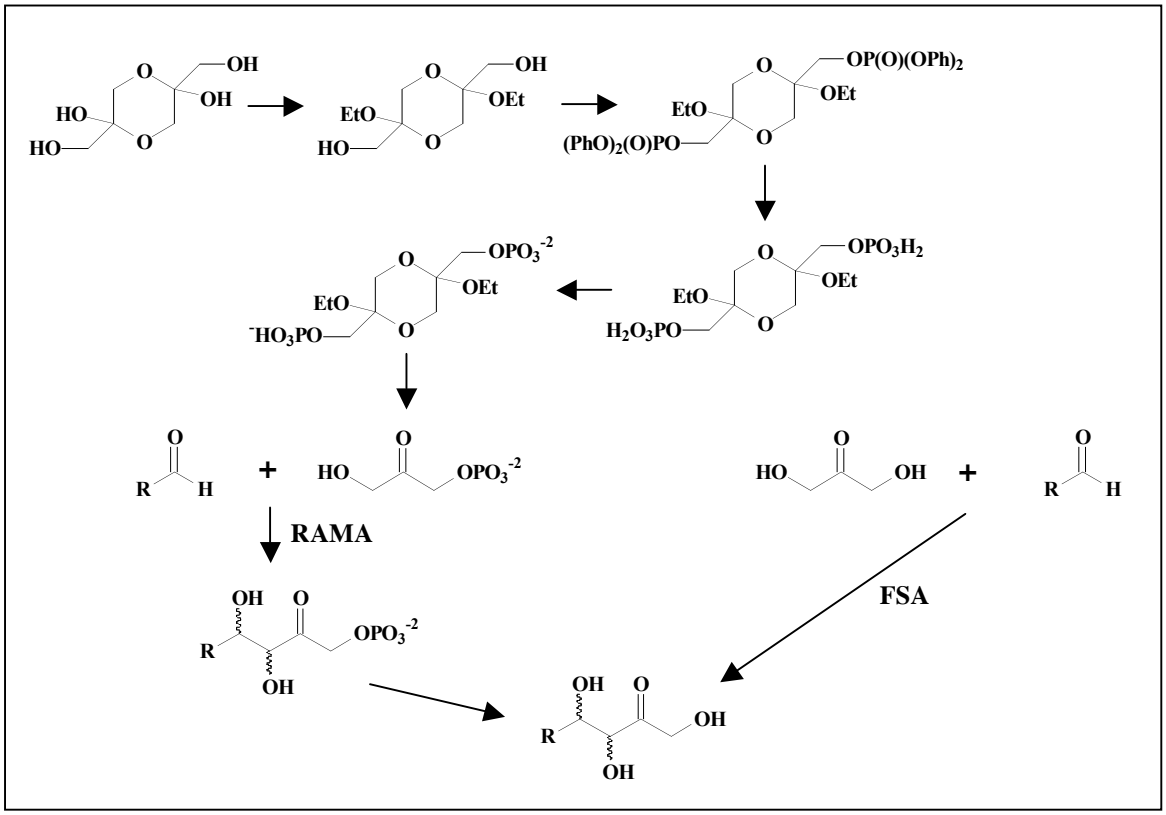


Fig.4-1. Preparation pathway of the aldol adduct with FSA using DHA or with RAMA after synthesis of DHAP (performed by J.A Castillo and P. Clapes (CSIC, Barcelona))

It is advantageous to perform the reaction at 4°C with a view to avoiding side reactions of aldehydes (inhibition of FSA) that are chemically aggressive. A fed-batch reaction system would a solution to minimize unfavorable reactions. In the system, aliquots of aldehyde will be supplied into the reaction mixture intermittently up to the same amount of donor substrate (Bongs et al., 1997).

In conclusion, FSA and DHA as donor can be applied not only to the utilization of simple aldehydes but also to the reactions creating various types of monosaccharides or derivatives including N-containing sugars. FBP aldolases such as RAMA and the substrate DHAP can be replaced by FSA and DHA.

4-2-3. Expansion of the synthetic ability of FSA

FSA A129S was highly active in the reactions using DHA as a donor substrate. This can enlarge the utility of FSA. The mutant yielded larger amount of *S*-erythrulose than WT did, and showed more than 50% of conversion rates in D-xylulose production from DHA+glycolaldehyde at 24h, whereas those of WT were less than 10%. This indicates that FSA A129S can be applied to various other reactions with DHA and produce larger quantities of unique and valuable sugars or sugar phosphates than WT does. However, it appeared that there was no difference between WT and A129S in catalytic ability for a reaction using hydroxyacetone as donor. (Residual hydroxyacetone concentrations and the areas of a new product in HPLC were almost same in both enzymes.) The reason can be assumed as the following by comparison of models of the active centers (Fig.4-2). A129S creates a hydrogen bond with DHA at Ser129 residue (Fig.4-2a), whereas WT does not have the bond due to Ala at the corresponding residue (Fig.4-2b). It is supposed that the hydroxyl group of S129 creates a hydrogen bond with a hydroxyl moiety at C1 atom of DHA. Thus, this could enhance the affinity to the substrate and improve reactivity in A129S. On the other hand, when hydroxyacetone is utilized as a donor substrate, a methyl moiety of hydroxyacetone would come into close proximity of S129 residue instead of a hydroxymethyl moiety of DHA. Thus, FSA A129S may not form the hydrogen bond by Ser129 residue (Fig.4-2c). As a result, there was no difference in yielding the product between A129S and WT, the same reactivity was observed in both enzyme reactions.

Two reactions (hydroxyacetone+glycolaldehyde with WT and DHA+glycolaldehyde with A129S) were achieved at 4°C, the conversion rates at 24h were almost equivalent

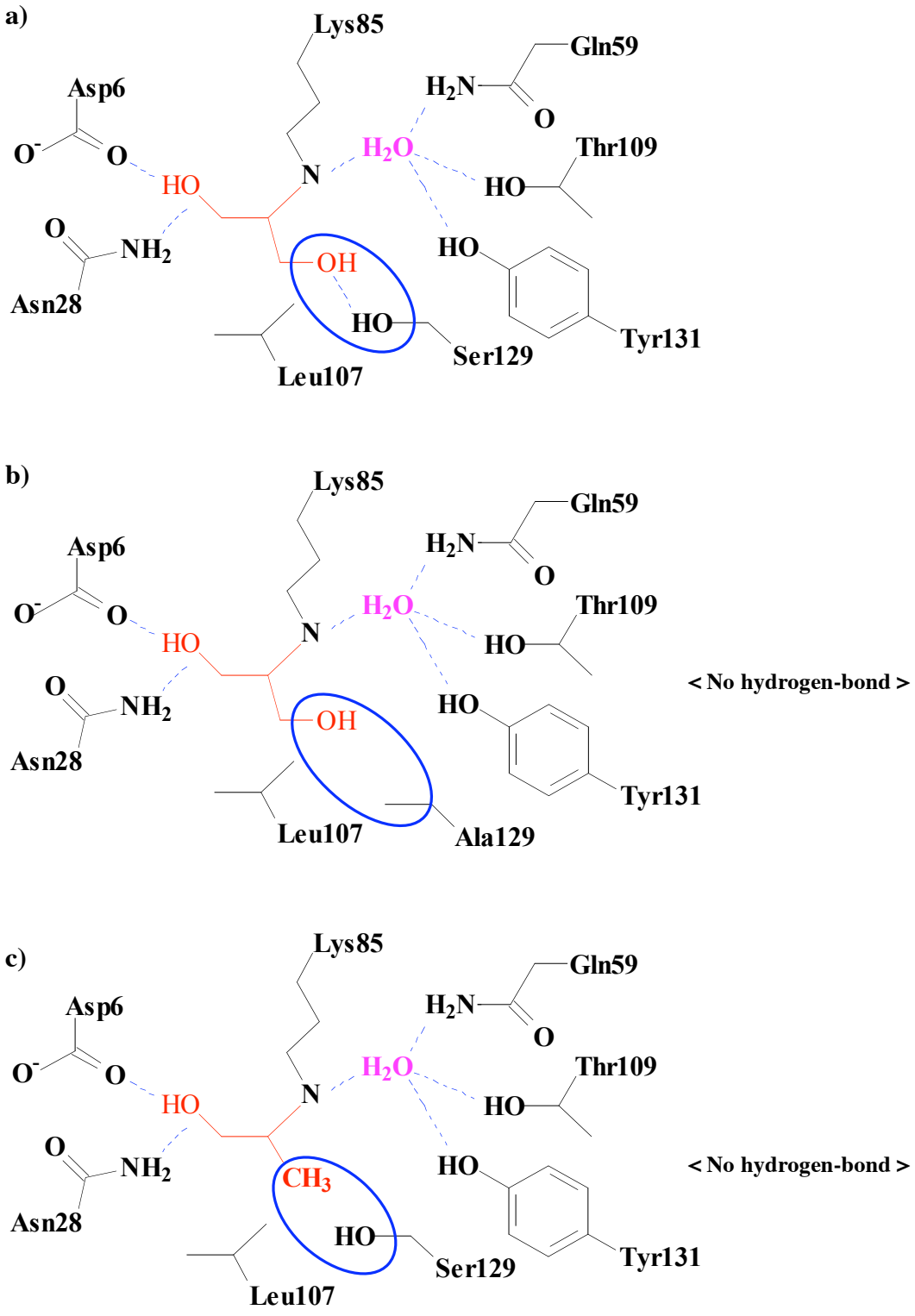


Fig.4-2. Two-dimensional models of active center a) of FSA A129S with DHA, b) of FSA WT with DHA, c) of FSA A129S with hydroxyacetone(HA), forming Schiff-base with a lysine residue (Lys85).

to those of reactions at 30°C in both cases. This implies that FSA could be a useful catalyst in synthetic applications. Actually, the utilization of enzymes at low temperature may contribute to extension of their lifetime or stabilization of substrates. Otherwise, the application at ambient temperature would simplify reactors and save both initial and running cost.

4-3. Evaluation of mutagenesis using error prone PCR

By error prone PCR with a mixture of two polymerases, random mutagenesis was achieved and various FSA mutants were obtained. Regarding nucleotides alteration, there was a difference in frequency of alteration among DNA bases (Table.4-3). The exchange between A and G was remarkable, the alteration into T from others was preeminent.

Table 4-3. Frequency of alteration of each base in *fsa*.

A → G	12		G → A	17	
A → T	9		G → T	10	
A → C	2		G → C	0	
	⁄ 23			⁄ 27	
T → A	7		C → A	5	
T → G	2		C → G	3	
T → C	5		C → T	12	
	⁄ 14			⁄ 20	
G,T,C →	A	29	A,T,C →	G	17
A,G,C →	T	31	A,G,T →	C	7
A; +6		G; -10	C; -13		T; +17

The amount of altered positions is 84 in 27 samples. The last part indicates calculated alteration balances of DNA bases.

By comparison of each base in the amount of frequency number, T had small magnitude of change into others (14 locations) but large from other bases (31 locations). On the other hand, C was not converted from other bases (only 7 locations), G also had lower frequency of alteration from other bases. A was highly active in exchange from and into others. Consequently, the balance of alteration clearly showed the difference of each DNA base. T was relatively much increased in the number (+17), both G and C were decreased apparently (G; -10, C; -13). Mutational spectra data by Stratagene is the following (Table.4-4) and the conversion into A and T is vigorous but not so into G or C as well as this experimental data in this work.

Table.4-4. Mutational spectra of nucleotides with a mutagenesis Kit (Stratagene, 2003).

(Transitions)			
	A -> G, T -> C ; 17.5%		G -> A, C -> T ; 25.5%
(Transversions)			
	A -> T, T -> A ; 28.5%		A -> C, T -> G ; 4.7%
	G -> C, C -> G ; 4.1%		G -> T, C -> A ; 14.1%
(Insertions and Deletions)			
	Insertions ; 0.7%		
	Deletions ; 4.8%		

The enzyme in the kit was a blend of two mutant polymerases.

Turning to amino acid alteration, various residues were mutated at random positions (Fig.4-3), it is expected that different forms of mutants will be acquired with this method. Actually, obtained 26 mutant proteins had variation with the alteration of 1 to 5 residues or creating stop codon (1 residue alteration: 6 samples, 2 residues: 7 samples, 3 residues: 7 samples. 4 residues: 3 samples, 5 residues: 1 sample, creation of stop codon: 2 samples ; Table.3-9 in Result). Eight samples of those were assayed with a usual photometrical method further, and some of them showed different or contrary results from those with multi-well assay using NBT and diaphorase. The reason is unclear yet, however, it is supposed that several compounds that could be reducing power for NBT might be contained in cell-free extracts.

Mutant FSA V88M obtained with error prone PCR showed interesting aspect. It retained lower activity than WT and was denatured by heat treatment as well as mutant Q59E. The residue V88 does not appear to be crucial for activity or contribute to stabilize the whole structure because the position is between β4 strand and α4 helix. Furthermore, the corresponding residue of V88 is methionine in some TALs (e.g. TAL B from *E.coli*, *B.subtilis*), leucine (*Rhodobacter capsulatus*, *Deinococcus radiodurans*) or valine ("TAL C" from *E.coli*, *Yersinia pestis*) (Schürmann Me, 2001). So it was expected that the alteration from valine into methionine could give little influence.

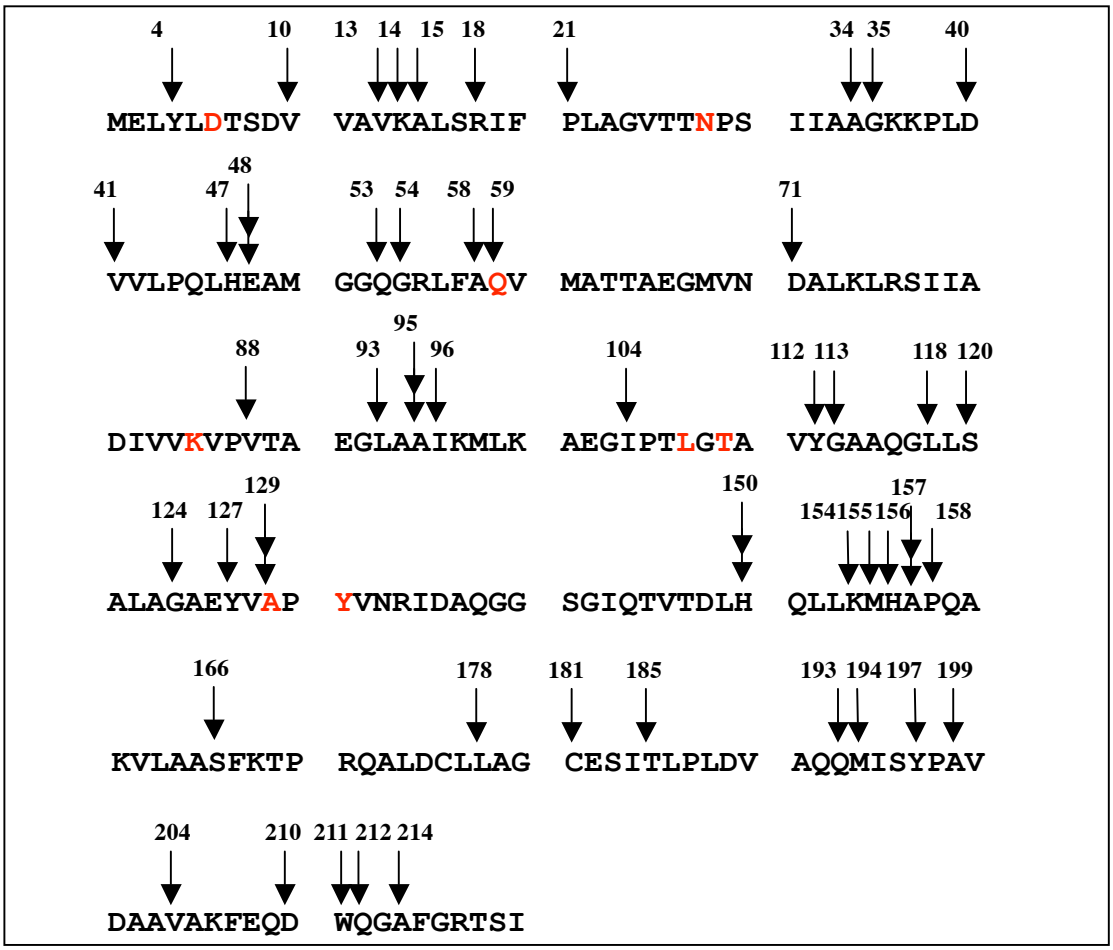


Fig.4-3. Amino acid sequence of FSA and exchanged positions (arrows and numbers) in 27 samples obtained by error prone PCR: Double arrows show that the alteration was observed at the amino acid in 2 different samples. Letters colored by red indicate amino acid residues at the active center.

However, this changing probably might deprive FSA of an appropriate conformational balance and make the enzyme unstable. Methionine has a longer side chain than that of valine, it is not branched and a sulfur atom is contained, those differences can generate unfavorable imbalance for FSA.

Four mutants (Q59H-H150Y, I96V-A129T, Y112N-G124D-A129G-D210E, ORF encoding 110 amino acids) had lower or no activity, no corresponding protein band was recognized for 3 samples with SDS-PAGE (Y112N-G124D-A129G-D210E, ORF encoding 110 amino acids and ORF encoding 52 amino acids). Those proteins may be unstable and therefore would be degraded in cells. Two mutants at 2 residues, Q59H-H150Y and I96V-A129T, contain alteration of residues at the active site (Q59, A129). The change of Q59 into histidine or of A129 into threonine might bring critical problems in activity. Both K37R and H47L that were mutated at single residue retained approximately full activity of what WT showed after heat treatment. Those alterations would not give drastic change to the enzyme, probably because they are located on α2 helix or in the vicinity of it that is some distance from the active center.

Mutants including alteration at more than 2 sites or ORFs encoding smaller size of proteins than FSA by appearance of stop codon may be inactive and make it difficult to understand the structural-functional relationship of the enzyme (Brannigan et al., 2002; Dalby, 2003; Tanaka et al., 2005; Tsuji et al., 2001; Williams et al., 2003). Those mutations are undesirable in experiment and actually improper to evaluate the mutants. Thus, it should be required to control the frequency of modification per gene. In fact, the quantity of template DNA and the reaction cycle number can be considered to decrease the number of alteration per gene, it can be suppressed by preparation of larger amount of template DNA or giving low cycle number of PCR (Stratagene, 2003). Once interesting mutants were obtained, such samples can be utilized as templates for error prone PCR further to get double or more point mutation. For instance, FSA A129S would be used as template and it could be expected to obtain mutant enzymes that keep high activity but acquire other stereoselectivity. This experimental result demonstrated that error prone PCR could be a useful technique for creating enzyme libraries that were composed of various forms of mutant proteins.

4-4. Comparison of TAL_{Bsu} with FSA in His-tag modification

His-tagged TAL_{Bsu} was prepared with both N- and C-terminal and the binding to

Ni-NTA superflow column was investigated like FSA. Interestingly, N-terminal His-tagged TAL_{Bsu} had no affinity to the resin, while C-terminal His-tagged one bound to it, as is the case with FSA. N-His-tagged enzyme retained higher activity than C-His-tagged one as well as N-His-tagged FSA had shown, though His-tag had a negative influence to TAL_{Bsu} and both His-tagged TAL_{Bsu} held lower activity than His-tagged FSA. It is supposed that the resemblance in monomeric structure between FSA and TAL_{Bsu} may lead to the similarity in results. On the contrary, whereas both His-tagged FSAs retained decamer structures, quaternary structures of TAL_{Bsu} were altered and fusion proteins became a dimer (N-His-tagged) or a mixture of dimer and pentamer (C-His-tagged). It is one of the noteworthy characteristics of TAL_{Bsu}. However, it is not clear yet, how they form dimer or pentamer and why they lost activity. TAL B from *E.coli* retained activity after monomerization of the quaternary structure in pH8.5 by alteration of a residue R300 that contribute to the dimerization (Jia, 1997; Schörken et al. 1998). Crystallization and 3-dimensional analysis of the His-tagged TAL_{Bsu}s could help to elucidate the structural characteristics of TAL_{Bsu} and to make the difference from dimeric TALs clear.

4-5. Similarity of TAL_{Bsu} to FSA in structure and difference in reaction

Analysis of TAL_{Bsu} crystal revealed it is a homodecameric protein and it has quite similar both monomer subunit and quaternary structure to FSA, in spite of having the shorter C-terminal α helix (Fig.4-4) (analysis by T. Sandalova). TAL_{Bsu} held a catalytic water molecule at the same location as that in FSA. Those two proteins have high similarity in structure but catalytic reactions are different each other. In fact, TAL_{Bsu} requires an acceptor substrate with Fru6P as donor (no activity for the cleavage of Fru6P) and showed low activity for Fru6P formation (DHA+GAP -> Fru6P: 0.01 U/mg). In this respect, it is supposed that TAL_{Bsu} and FSA have a different way to stabilize the intermediate. Transaldolases can stabilize and hold the intermediate of first substrate better than FSA, therefore the intermediate may wait till a second substrate is set at the active site of transaldolases, whereas FSA releases it as DHA with GAP. The variety of some residues surrounding a catalytic water molecule such as Glu58(TAL_{Bsu}) - Gln59(FSA) and Phe130(TAL_{Bsu}) - Tyr131(FSA) gives rise to inquiries how this water molecule can be held at the position or if there is a difference for retaining it and for transfer of electrons via the molecule in both enzymes. The existence of Tyr131 appears

to be essential to keep the activity in FSA, while TAL_{Bsu} has Phe and it doesn't seem to have any function to participate in the reaction directly. When the carboxyl moiety of Glu is compared with the amido group of Gln, the former can bind more strongly to the water molecule due to the difference of electronegativity between oxygen atom and nitrogen. This may generate different stability to hold the water molecule or facilitation of proton abstraction in electron transfer. Preparation and assay of a double mutated FSA Q59E-Y131F should give some hints to the idea. If it would retain the activity, Glu58 in TAL_{Bsu} could play the same role as Y131 in FSA despite of being unstable. In stability, both FSA Q59E and Y131F were denatured by heat treatment. TAL_{Bsu} was occasionally denatured after heat treatment at 75°C for 40min, and therefore the manipulation required to be stopped in 30min for that protein.

J. A Castillo & P. Clapes tried to apply TAL_{Bsu} to the reaction with an aminoaldehyde (*N*-Cbz-3-aminopropanal) as well as FSA, however, the product was not detected. The reactivity of DHA by TAL_{Bsu} was about 10% of that of Fru6P (Schürmann Me, 2001, data in this study). The active center of TAL_{Bsu} does not appear to be so flexible for acceptor substrates as that of FSA and the aminoaldehyde cannot be adopted to TAL_{Bsu} easily.

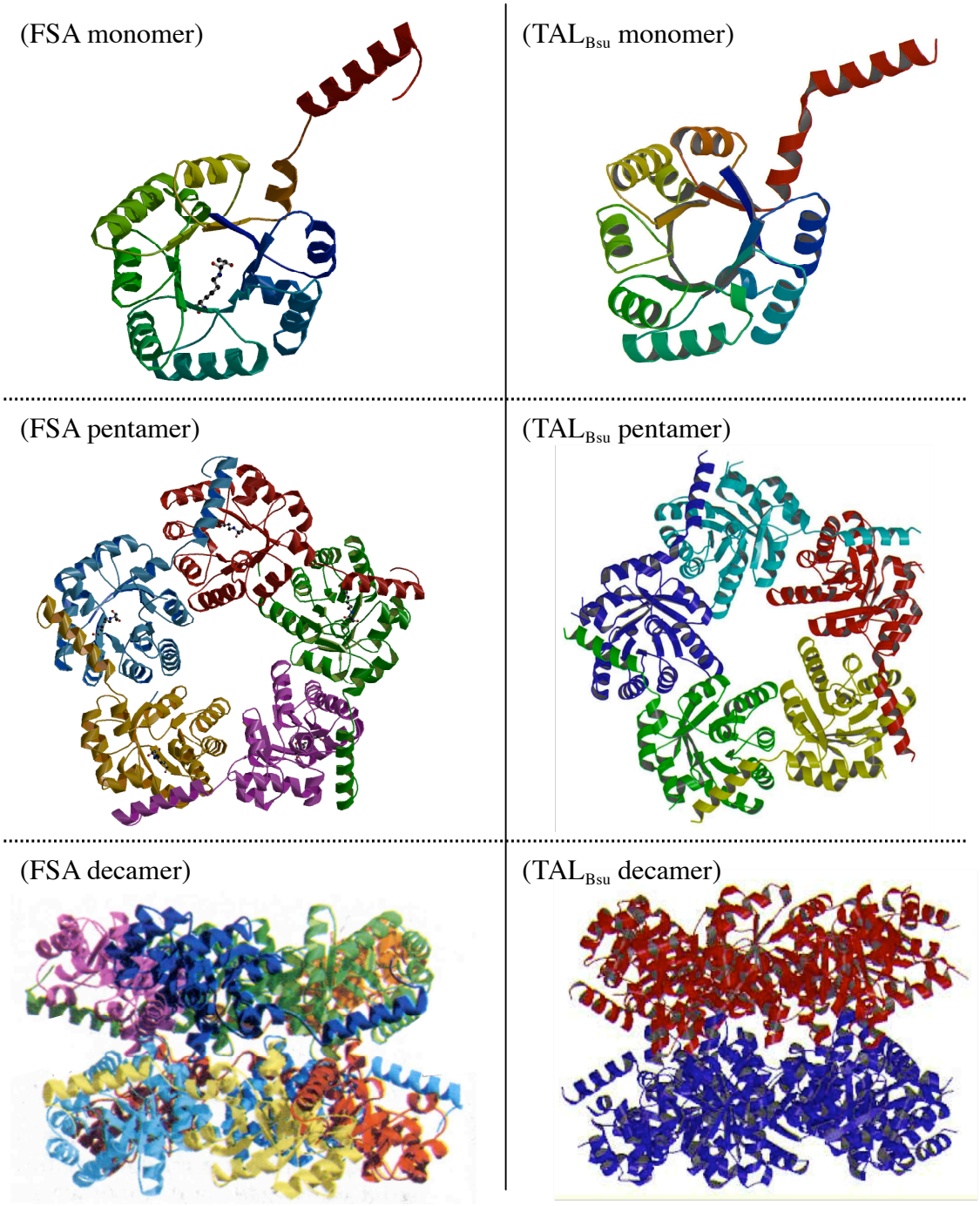
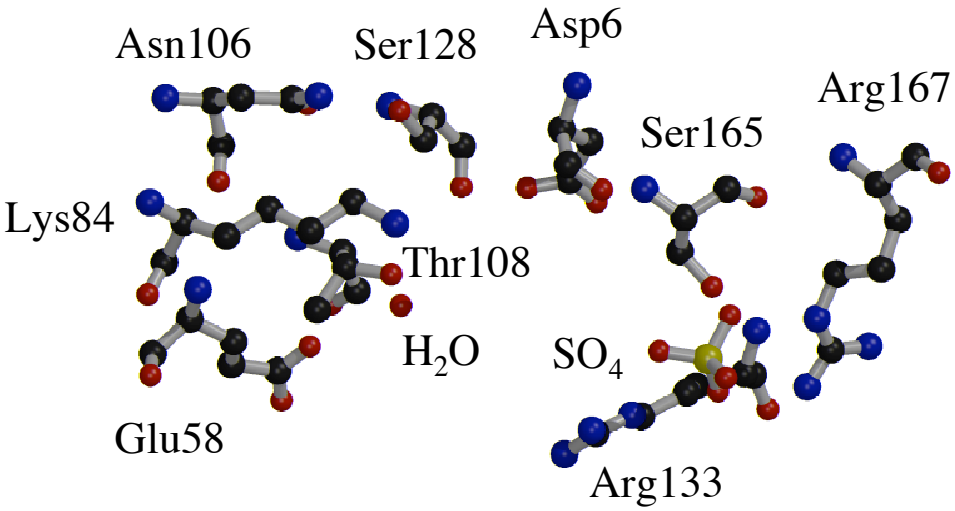


Fig.4-4. Comparison of FSA with TAL$_{Bsu}$ in structure; C-terminal helix of TAL$_{Bsu}$ in the monomer subunit is shorter than that of FSA. Both pentamer form have a star or doughnut shape, decamers result from dimerization of pentamers (3-dimensional analysis for FSA; S. Thorell et al., 2002, for TAL$_{Bsu}$; data from T. Sandalova).

a) an active site model of TAL_{Bsu} with SO_4



b) a model of the active center of TAL_{Bsu} bound to arabinose-5P

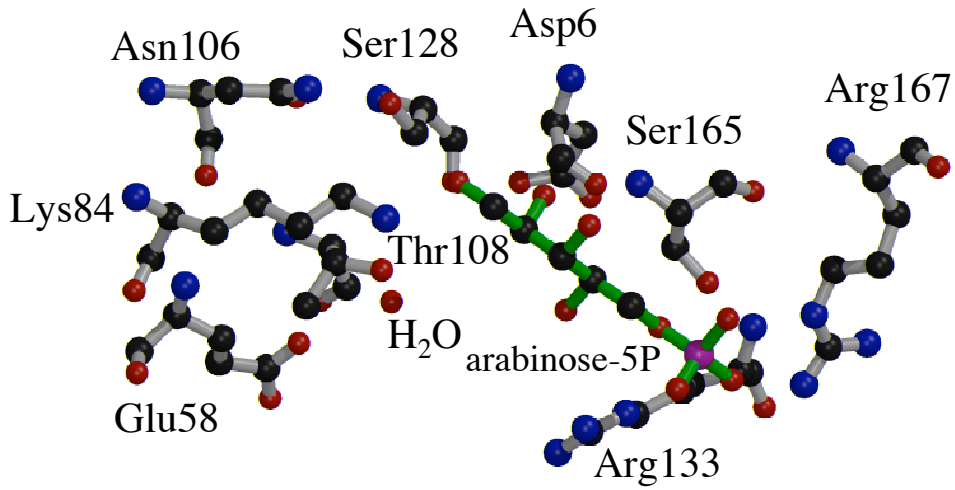


Fig.4-5. Active site models of TAL_{Bsu} with a) SO_4 or b) arabinose-5P (data courtesy of T. Sandalova, Karolinska Institute, Stockholm); black ball- C atom, blue- N atom, red- O atom, yellow- S atom. A green stick represents the arabinose-5P bone structure.

The enzyme substrate complex models by T. Sandalova indicate that phosphate binding sites of the enzyme may be rather rigid and a phosphate group of all substrates can bind to the same residues. Actually 4 different complex models, I.TAL_{Bsu}- Ara5P, II.TAL_{Bsu}- Fru6P, III.TAL_{Bsu}- DHA intermediate (carbanion) + Ery4P and IV.TAL_{Bsu}- DHA intermediate (carbanion) + GAP were examined for the conformity, Fru6P in model II fit the active site perfectly and GAP in model IV was located further from carbanion than Ery4P was in model III. Those models were dependent on the hypothesis that the position of acceptor substrates at the active center would be determined by the binding of a phosphate group of substrates to amino acid residues of TAL_{Bsu}. Practically it is expected that enzymes would have flexibility to some extent.

Focusing on the residues that bind to a phosphate group of substrates in TAL_{Bsu}, three residues, R133, S165 and R167 are found in TAL_{Bsu} (Fig.4-5), R133 and S165 are absolutely conserved in not only "FSA class" but also any other TALs. In FSA, those residues, R134, S166 and K168 instead of R167 are recognized. The position of a guanidium group of R167 in TAL_{Bsu} is replaced by the side chain of N133 in FSA. TAL_{Bsu} has glycine (G132) at the corresponding position and does not have the appropriate asparagine for it. It means that FSA may have four ligands for the phosphate group, the trapping manner of substrates is not identical between TAL_{Bsu} and FSA. This may bring different substrate specificities to them.

Multiple alignment analysis of amino acid residues at the active center of FSA and transaldolases by T. Sandalova suggests a classification of the enzymes into 2 classes that can be useful to understand the difference of affinity to substrates. Each type has similar amino acid sequence for 9 residues to either TAL_{Bsu} or FSA (Table.4-5).

Table.4-5. Classification of TALs by the alignment of residues at active center

a.a. position*	58	106	128	130	132	133	165	167
TAL_{Bsu} type	Glu	Asn	Ser	Phe	Gly	Arg	Ser	Arg
FSA type	Gln	Leu/Thr	Ala	Tyr	Asn	Arg	Ser	Arg/Lys

*The numbering of amino acid residues is based on that of TAL_{Bsu}.

Corresponding positions for FSA are added by +1.

4-6. Comparison TAL_{Bsu} and FSA with other aldolases

Further comparison of amino acids sequences or protein structures may give some clues to interpret the characteristics of FSA type aldolases or transaldolases (TAL_{Bsu} type). Five enzymes, FSA (FSA A), "TAL C" from *E.coli* (FSA B), *Bacillus subtilis* (TAL_{Bsu}), TAL from *Thermotoga maritima* (TAL_{Tm}) and TAL from *Methanocaldococcus jannaschii* (TAL_{Mj}) were compared in their identities of amino acids sequence (Table.4-6) and the alignment (Fig.4-10). FSA A and FSA B do not have TAL activity, the others possess TAL activity lacking of FSA activity by nature (Schürmann & Sprenger, 2001). They are divided into 2 groups from the result of Table. 4-5, which corresponds to their catalytic functions mentioned above (FSA or TAL reaction). Three TALs (TAL_{Bsu}, TAL_{Tm}, TAL_{Mj}) are identical by approximately 30% to FSA A, TAL_{Tm} and TAL_{Mj} have more than 50% of identity to TAL_{Bsu}. On the contrary, FSA B is identical to FSA A by 70% but to TAL_{Bsu} by 35%.

Table.4-6. Identity of amino acid sequences for 5 enzymes and the reaction type

	TAL_{Bsu}	FSA B	TAL_{Tm}	TAL_{Mj}
to FSA A	66/212 - 31%	154/220 - 70%	65/218 - 30%	70/217 - 32%
to TAL_{Bsu}	100%	76/220 - 35%	119/218 - 55%	124/217 - 57%
reaction type	TAL	FSA	TAL	TAL

The residues shown on Fig.4-6 can be classified into 5 groups, 1-. conserved residues in all five enzymes, 2-. conserved in four of five enzymes, 3-. different residues between FSA type and TAL type, 4-. variance between FSA type + TAL_{Bsu} and the other two enzymes, 5.- non conserved residues. The determinant to distinguish FSA type aldolase from transaldolases can be the residues in third group, 37 residues are found in the group. Some of them are positioned at the active center, and those can be crucial to determine the reaction type. For instance, FSA mutant V88M does not appear to give drastic influence to the variation, actually this mutant enzyme still retained FSA activity. Residues Asp6, Asn28, Gln59, Lys85, Leu107, Thr109, Ala129 and Y131 in FSA are located at the active site and those are related to binding to DHA. Four residues, Asp6, Asn28, Lys85 and Thr109 are absolutely conserved in both types and the others are different between FSA type and TAL type.

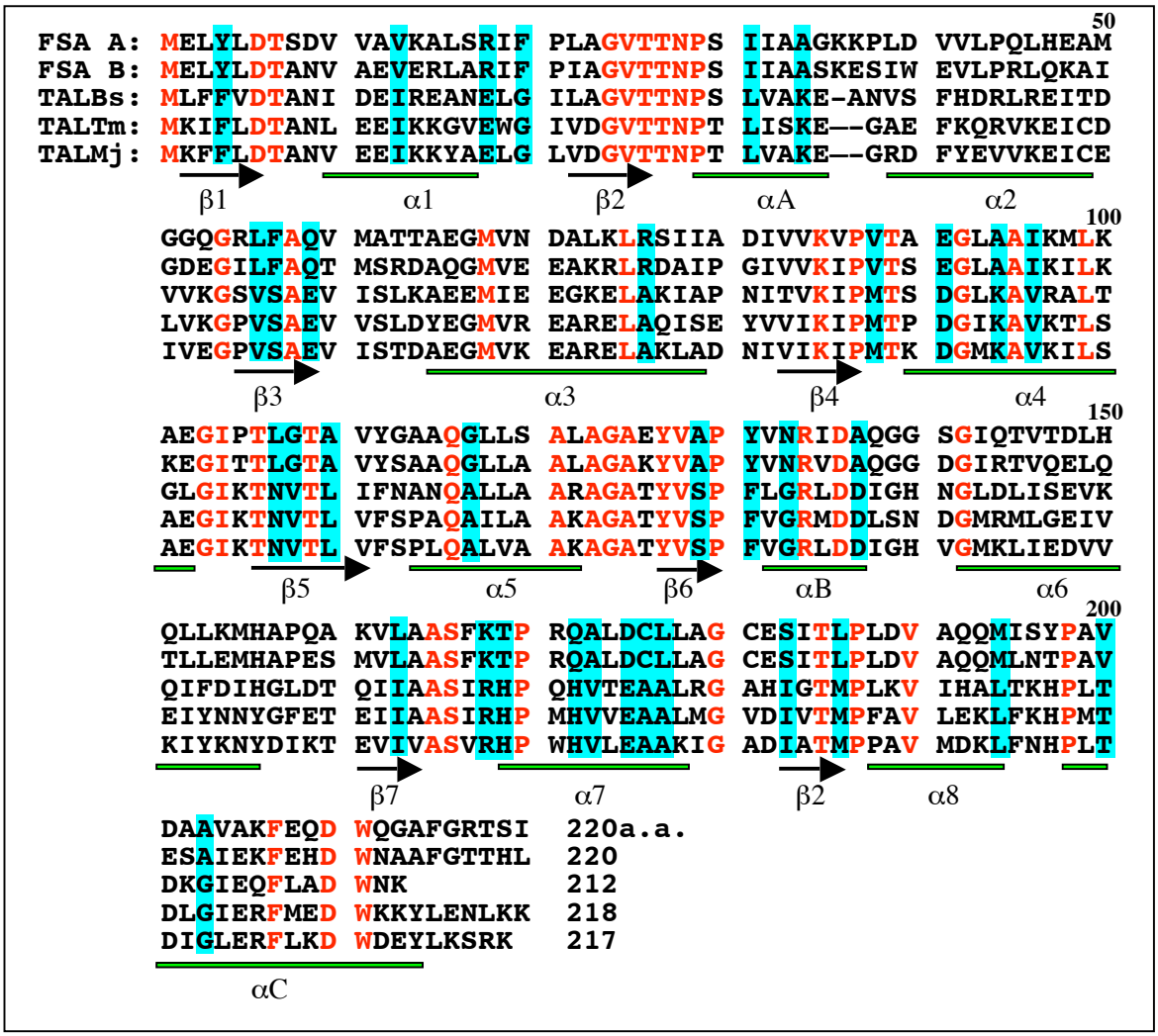


Fig.4-6. Structure based sequence alignment of transaldolase family (Schürmann, 2001, Thorell et al., 2002); FSA A: *E.coli* fructose 6-phosphate aldolase, FSA B: *E.coli* "TAL C", TALBs: *B.subtilis* transaldolase, TALTm: *T.maritima* transaldolase, TALMj: *M.jannaschii* transaldolase, black arrow; β strand, green bar; α helix. Red letters indicate conserved residues in 5 enzymes. Letters with blue backgrounds are residues discriminating FSA type from TAL type.

Residues Glu58, Asn106, Ser128 and Phe130 in TALs are corresponding to Gln59, Leu107, Ala129 and Y131 in FSA A or FSA B. According to the comparison of an active site model for FSA A with that for TALs, TALs appear to be able to stabilize and hold the carbanion more firmly by forming hydrogen bonds to hydroxyl groups of C1 and C3 as shown in Fig.4-7.

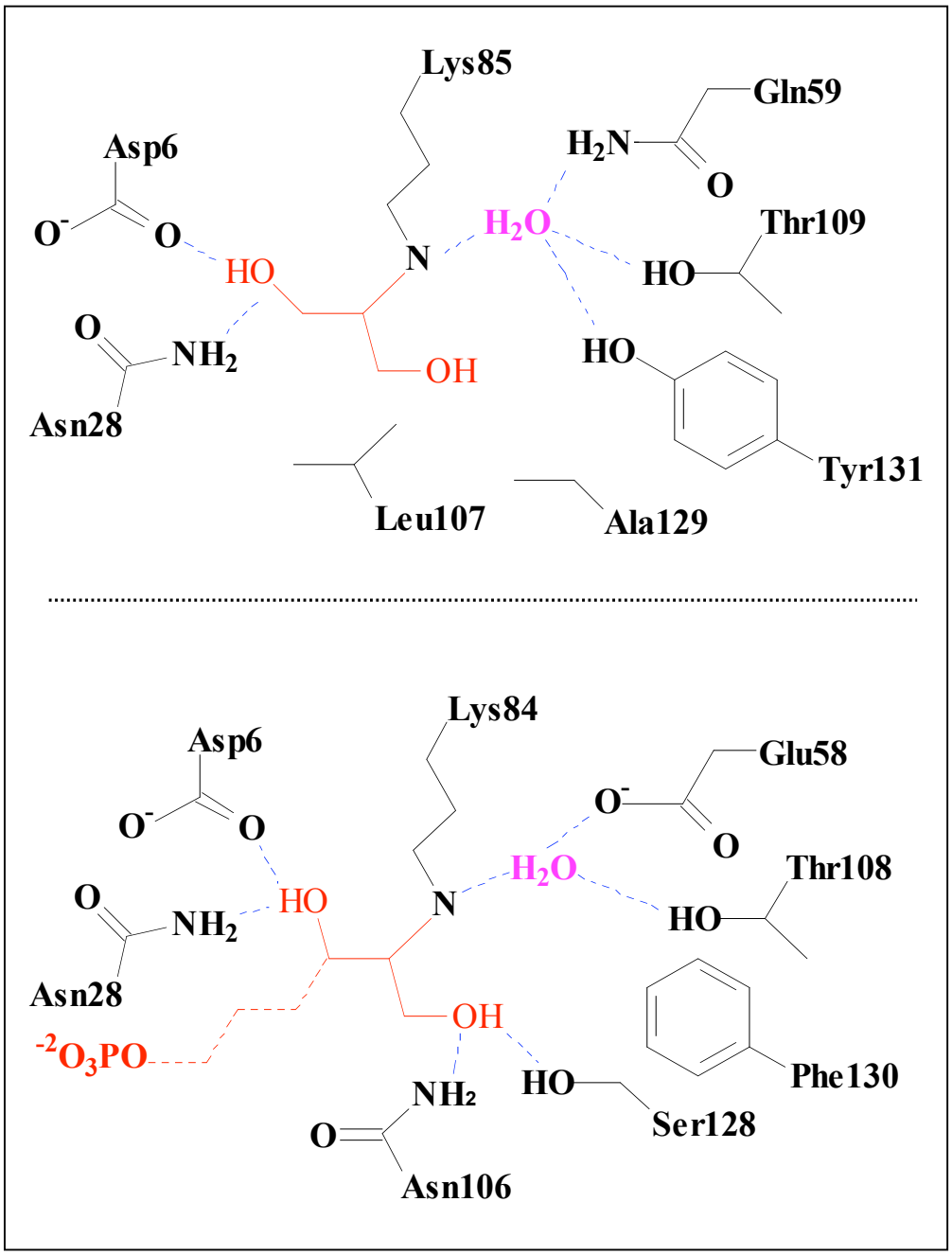


Fig.4-7. Two-dimensional models of Schiff-base forming active site for a) FSA A-DHA complex and b) TAL_{Bsu} –Fru6P complex. The number of hydrogen bond created with a water molecule or with a hydroxyl group at C1 of donor substrate are different between FSA A and TAL_{Bsu}.

However, a FSA mutant L107N/A129S prepared by Me.Schürmann showed almost same Vmax and lower Km for both DHA (in synthetic reaction of Fru6P) and Fru6P (in cleavage of Fru6P), those mutations cannot give critical change from FSA into TAL in the reaction. In this respect, additional alteration at Q59 and Y131 (a quadruple mutant; Q59E-L107N-A129S-Y131F) may bring some perspectives that the mutant would show TAL activity. At the same time, some residues such as Asn133 or A137 on αB helix which is a putative region binding to phosphate moiety of substrates can be taken into consideration for alteration. There is a convergence of residues classified above as third group on α7 helix, actually 6 of 10 residues are proper for the group. Some of them or Lys168 beside the helix may have distinctive roles to behave as FSA or TAL. However, the alteration at plural residues per gene may give imbalance for the structure and make the enzyme unstable or unfavorable for the investigation. Chimera enzymes might not keep the stability and it was difficult to produce those proteins, although they were composed of proteins that exist in nature. It implies that structural components as well as catalytic functions should be taken into consideration to design and create mutant proteins. In addition, there may be critical residues that are essential to form or retain the basic conformations of proteins, virtually even single residue alteration sometimes can bring instability of protein structures.

5. Summary

Goals of this work were the elucidation of structural-functional characteristics for fructose-6-phosphate aldolase (FSA) from *E.coli* and transaldolase from *B.subtilis* (TAL_{Bsu}), clarification of both enzymatic features, and evaluation of their synthetic abilities of the enzymes to produce valuable sugars or the derivatives. FSA and TAL_{Bsu} were overproduced in recombinant *E.coli*, purified with FPLC and assayed for their catalytic ability using several substrates. The following results were obtained:

1. Both N- and C-His-tagged FSAs were prepared and investigated in activity and quaternary structure to compare with mutant His-tagged FSAs. However, both His-tagged proteins lost activities (less than 50% residual) while retaining their quaternary structures.

2. FSA wild type (FSA WT) was purified with a new method by using both an anion-exchange column (Q-Sepharose) and a hydroxyapatite column after heat treatment (at 75°C for 40min).

3. Amino acid residues at the active site in FSA (Q59, Y131) were investigated for the activity by using site-directed mutagenesis. Mutant FSA Q59E was purified and crystallized. It was not changed in structure and retained 66% (6.4 U/mg at 30°C, pH8.5) of the activity shown with FSA WT (9.7 U/mg). Both Y131A and Y131F lost activities almost completely, though their quaternary structures were retained as decamer.

4. FSA and mutant A129S showed considerable synthetic potential and produced valuable sugars like *S*-erythrulose or D-xylulose using dihydroxyacetone (DHA) as donor and formaldehyde or glycolaldehyde as acceptor. Products were yielded at 4°C after 24h as much as those at 30 °C, which can extend lifetimes of enzymes and avoid side reactions of chemically aggressive substrates like aldehydes. FSA A129S had much higher activity (e.g. 0.21 U/mg for D-xylulose production) with DHA as donor than WT (0.01 U/mg), however, there was no difference in activity between both enzymes when hydroxyacetone (HA) was utilized as donor.

5. An aminoaldehyde (*N*-Cbz-propanal) was accepted and N-containing aldol adducts that can be precursors of antibacterial agents were prepared with FSA. The reactions also proceeded at 4°C. (This work was performed in cooperation with J. A Castillo and P. Clapes, CSIC, Barcelona)

6. Error prone PCR mutagenesis of FSA was performed. Various mutant FSAs at different amino acid residues were obtained and it was shown that the technical method was useful to prepare for a protein library.

7. Neither the N- nor C-His-tagged TAL_{Bsu} retained TAL activity or the original quaternary structure, whereas both N- and C-His-tagged FSAs kept their quaternary structures with the decrease of activities. In this respect, TAL_{Bsu} and FSA have different characteristics each other, and it is supposed that TAL_{Bsu} is not so stable as FSA in spite of their structural similarity.

8. TAL_{Bsu} wild type was purified with a new method (22.4 U/mg) by using both an anion-exchange column (Q-Sepharose) and a gel filtration column (Superdex 200pg) after heat treatment (at 75°C for 30min). Purified TAL_{Bsu} was crystallized by T. Sandalova at the Karolinska Institute (Stockholm), the 3-dimensional structure analysis revealed the enzyme was decamer and had high similarity to FSA in structure ($(\beta,\alpha)_8$-TIM-barrel).

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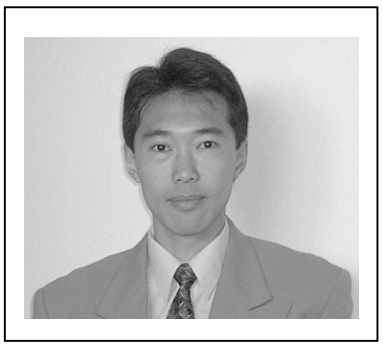

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