Enzyme Engineering

3. Thermodynamics and Stability of Enzymes

3.1 Protein Stability

3.2 Case Study 1: Enzyme Stabilization

3.1 Protein stability

Potentials & Bottlenecks of Enzymes

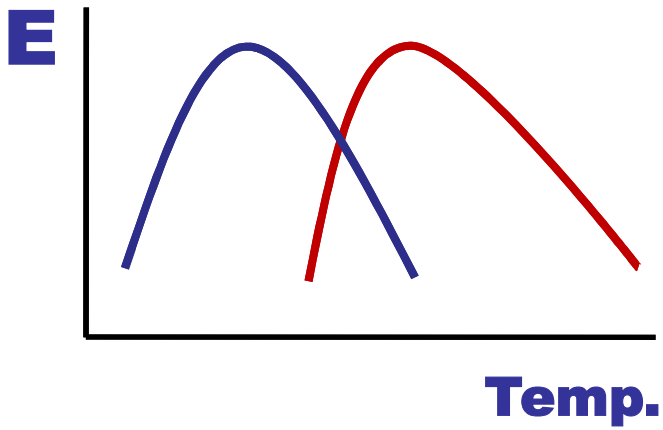
- **Bottlenecks**
  - Enzyme cost
  - Instability

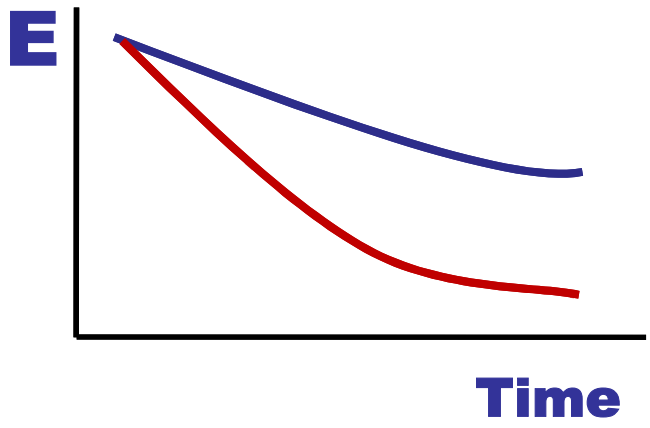
  * Enzymes are adapted to their particular function in living cells and they are therefore poorly suited for industrial applications (extremes of pH, temperature and salinity).
- **Potentials**
  - Substrate specificity
  - Mild reaction conditions

$$N \leftrightarrow U \rightarrow I$$

Unfolding Inactivation

- **Stability**
  - Thermodynamic (Conformational, Structural) stability
  - Operational (Kinetic) stability





Factors affecting stability

- Hydrophobic interaction
- Hydrogen bond
- Conformational entropy of unfolding
- Electrostatic interaction (salt bridge)
- Aromatic-aromatic interaction
- Disulfide bond
- …

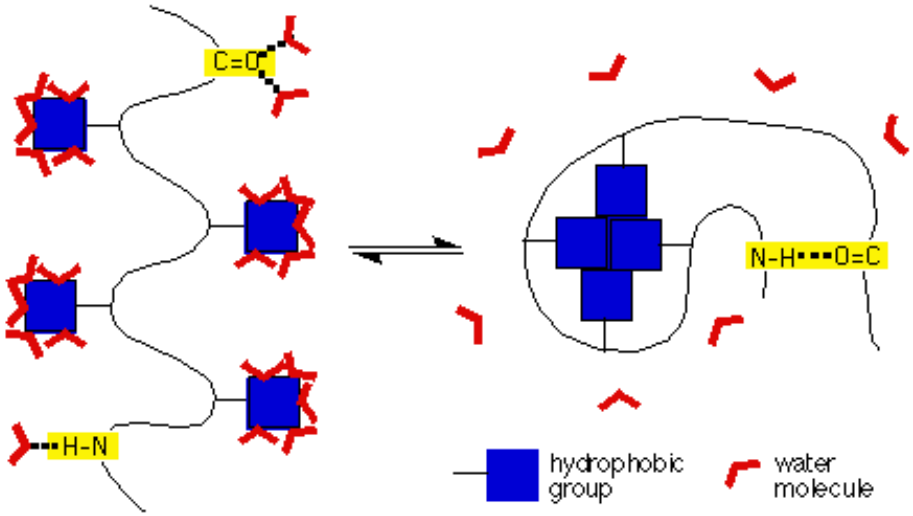
Factors affecting stability

- **Hydrophobic interaction**

  - The hydrophobic effect is considered to be the major driving force for the folding of globular proteins.

  - It results in the burial of the hydrophobic residues in the core of the protein.

  - The hyc ...uried methylene ...ol.



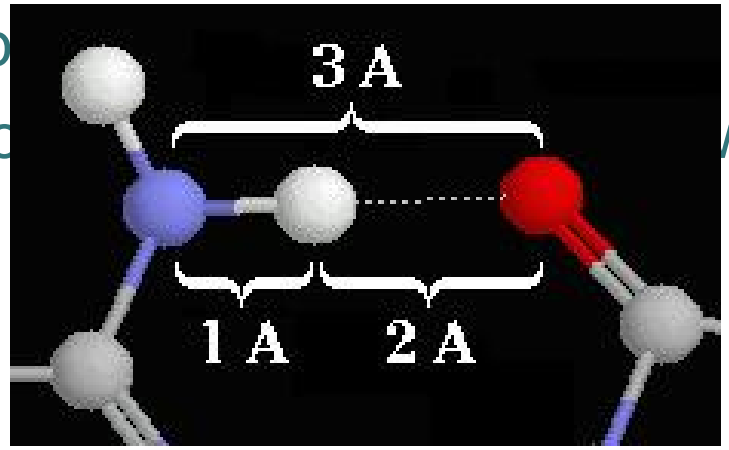
Factors affecting stability

- **Hydrogen bond**

  - A hydrogen bond occurs when two electronegative atoms, such as nitrogen and oxygen, interact with the same hydrogen.

  - The hydrogen is normally covalently attached to one atom, the donor, but interacts electrostatically with the other, the accep

  - The strength c ween 2 and 10 kcal/mol



Factors affecting stability

- **Conformational entropy of unfolding**

  - The factor that makes the greatest contribution to stabilization of the unfolded state is its conformational entropy.

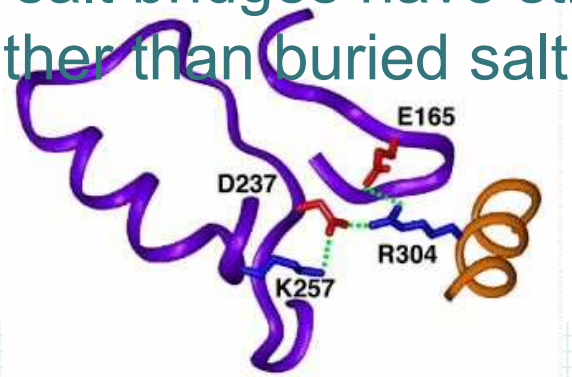
  - It has been proposed that decreasing the conformational flexibility of the unfolded chain (by substitution with proline, or by replacement of glycine) should lead to an increase in the stability of the folded relative to the unfolded protein.

Factors affecting stability

- **Electrostatic interaction (salt bridge)**

  - Salt bridges or ion-pairs are a special form of particularly strong hydrogen bonds made up of the interaction between positively charged residues (His, Arg, Lys) and negatively charged residues (Asp, Glu).

  - Salt bridges is a discriminating stabilization factor between thermophilic protein and mesophilic protein and especially surface salt bridges have strong stabilization effect to protein rather than buried salt bridges.



Factors affecting stability

- **Disulfide bond**
  - Disulphide bonds are formed by the oxidation of two cysteine residues to form a covalent sulphur-sulphur bond which can be intra or intermolecular bridges.
  - Calculations suggest that a disulphide bond should give rise to 2.5 - 3.5 kcal/mol of stabilization

Factors affecting stability

- **Aromatic-aromatic interaction**
  - Stabilizing interactions between two aromatic amino acids
  - The optimal geometry is perpendicular, such that the partially positively charged hydrogens on the edge of one ring can interact favorably with the pi electrons and partially negatively charged carbons of the other
  - About 60% of the aromatic side chains (Phe, Tyr, and Trp), found in proteins are involved in aromatic pairings.

Enzyme Stability: Exterior Factors

- Heat
- Organic solvents
- pH
- etc

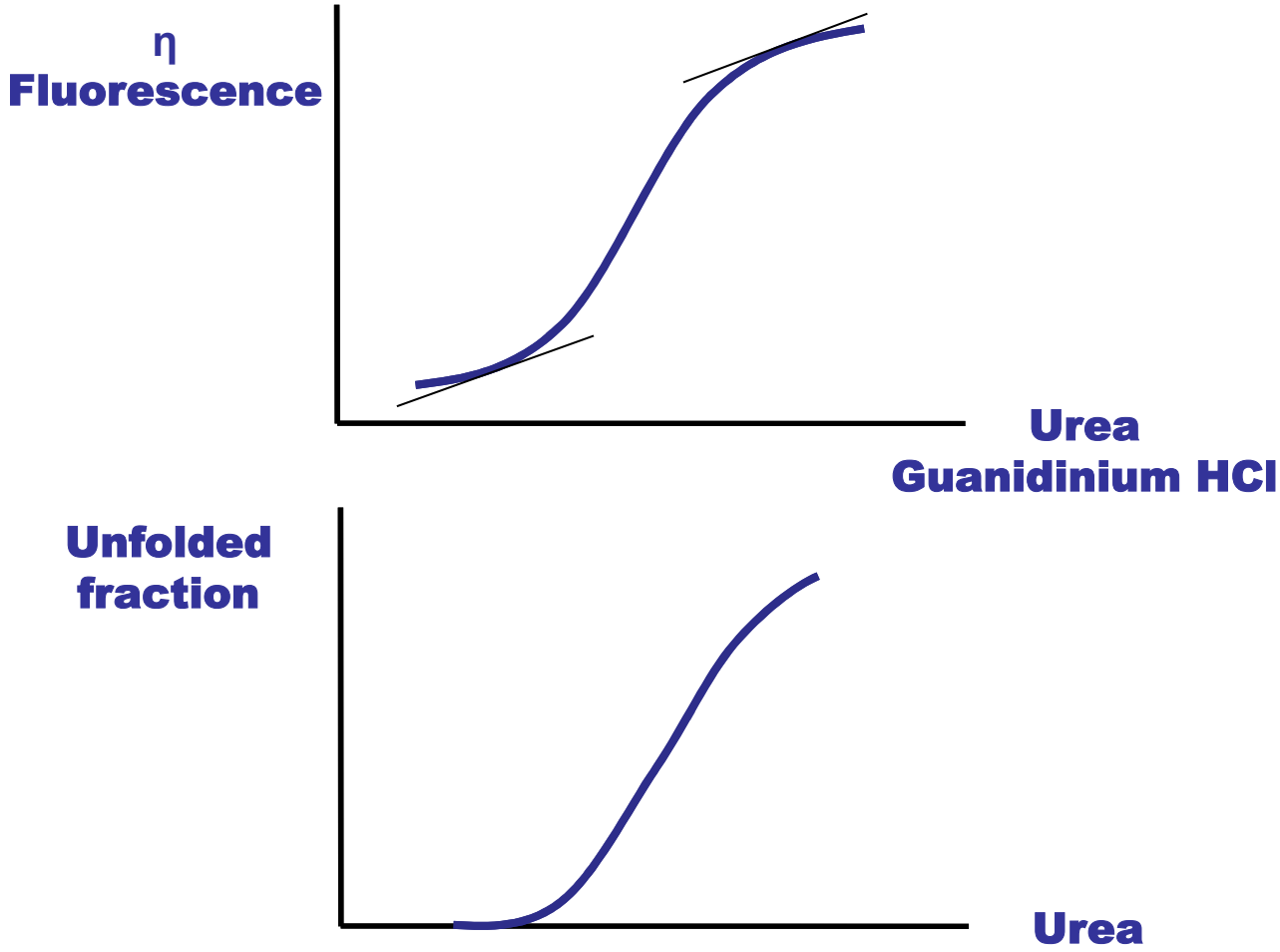
Measurement of folding stability

$$N \leftrightarrow U$$

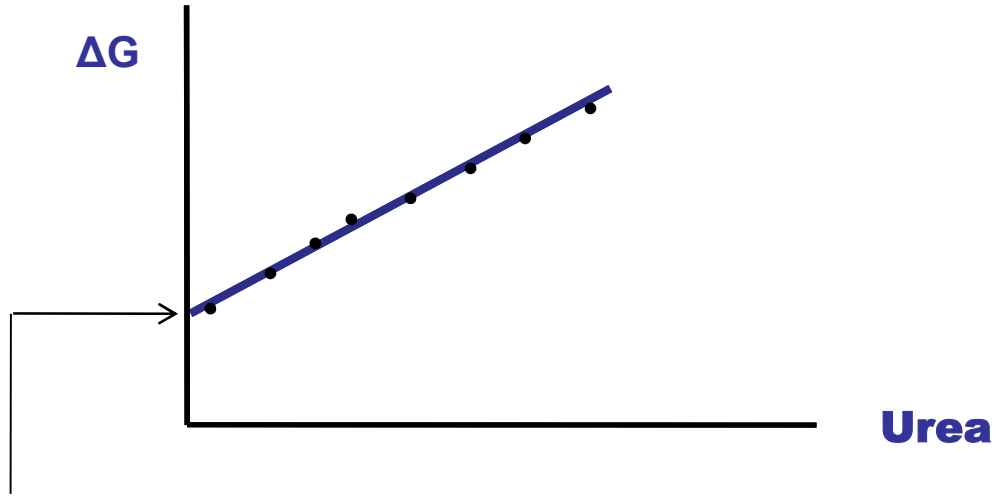
$$K = \frac{U}{N} = \frac{F_N}{1 - F_N}$$

$$\Delta G = - RT \ln K$$

Measurement of folding stability



Measurement of folding stability



ΔG in the absence of denaturant

Measurement of folding stability

- Tm : half unfolded melting temperature

  * Differential scanning calorimetry (DSC)

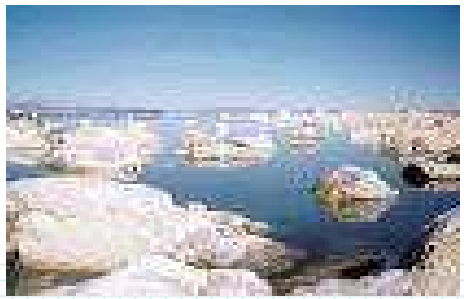
Approaches for enzyme stability

- Screening for novel enzymes
- Additives
- Immobilization
- Chemical modification
- Solvent engineering
- Protein engineering
  - Directed evolution
  - Rational design
  - Computational protein design

Approaches for enzyme stability

- **Screening for novel enzymes**
  - Screen microorganism from extreme environments (high temperature, high pH, high pressure, …)
  - Extremophiles have excellent functions and stability.
  - Lipase, xylanase, protease, α-amylase and DNA polymerase, … are used in industry.



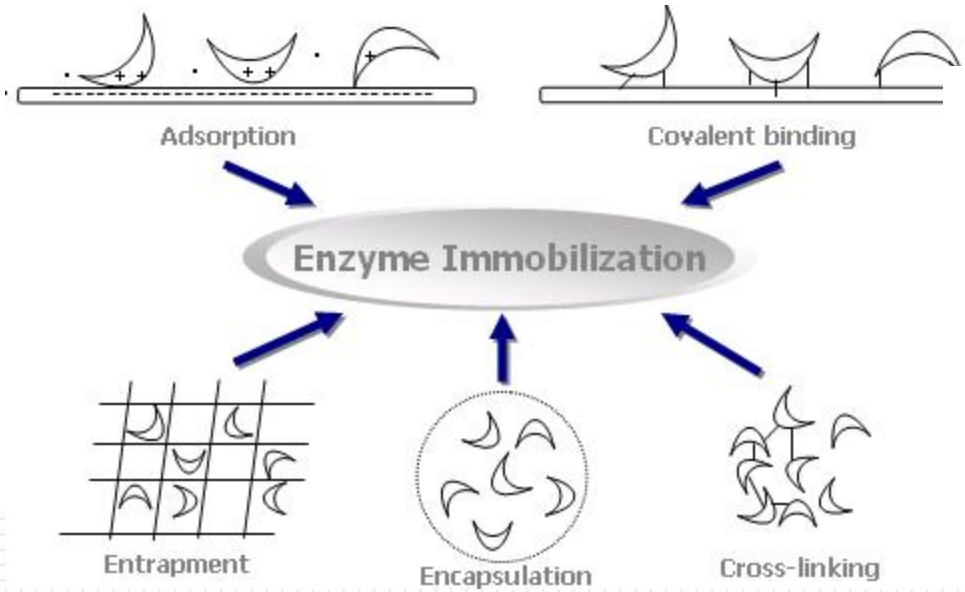


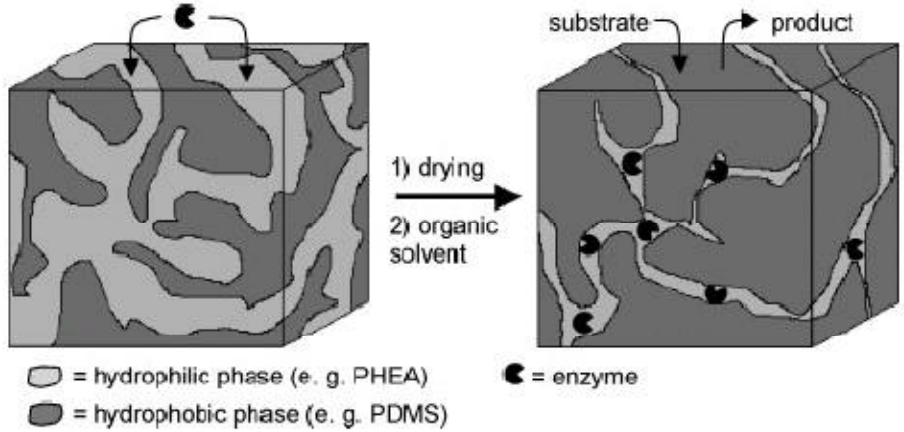
Approaches for enzyme stability

- **Additives**
  - Small molecules are added for stabilizing protein
  - A range of low-molecular weight additives exert stabilizing effects by inducing preferential hydration of proteins
  - Protein, amino acid, lipid, fatty acid, surfactant, metal, polyols

Approaches for enzyme stability

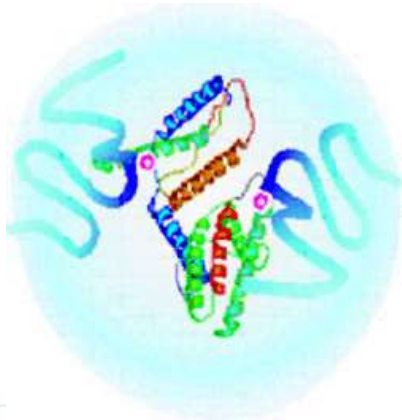
- Immobilization
  - To preserve protein stability and activity, protein is immobilized into support materials.
  - Immobilization method has long history and still remains effective tool to increase protein stability.





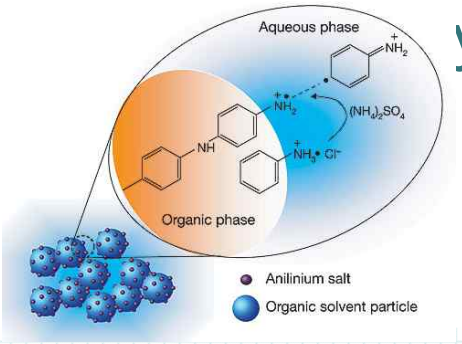
Approaches for enzyme stability

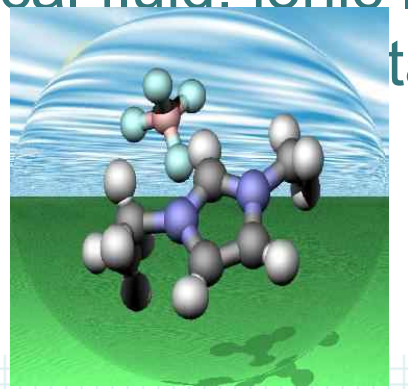
- **Chemical modification**
  - Chemical modification of amino acid residues R- groups remains useful for stabilizing enzymes
  - Crosslinked enzyme crystals, Covalent attachment polymers such as PEG, Combined site-directed mutagenesis and chemical modification approach,…

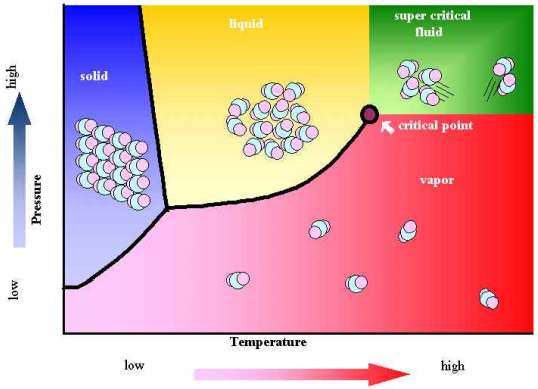


Approaches for enzyme stability

- **Solvent engineering**
  - Organic solvents as reaction media have many advantages and it has been widely investigated in academic and industrial field.
  - But organic solvents decrease protein stability and activity, solvent engineering such as control of organic solvent concentration, water activity, and selection of alternative media (supercritical fluid, ionic liquid) is needed to yme tabili







Approaches for enzyme stability

- **Protein engineering**

  - Most powerful tool to increase enzyme stability

    ① Directed evolution

    ② Rational design

    ③ Computational protein design

Approaches for enzyme stability

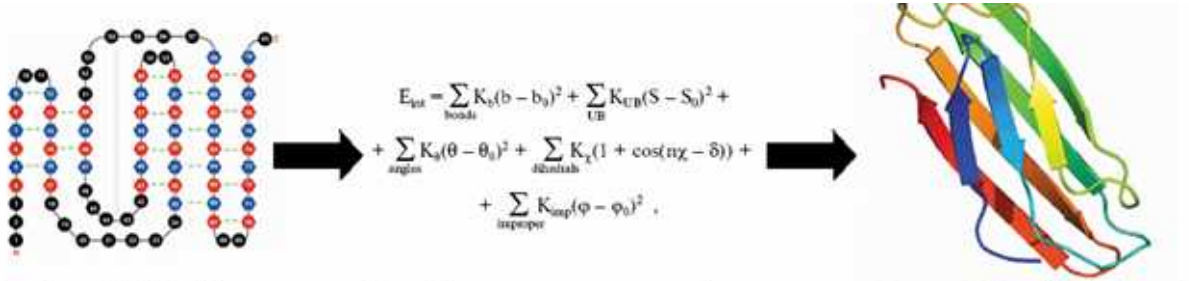
- **Directed evolution**
  - Directed evolution involves the recombination of beneficial point mutations with selection for further-improved properties.
  - The process can be repeated through successive cycles, leading to noteworthy alterations/improvements to the properties of the baseline protein.
  - No knowledge or modeling of the target protein's molecular structure is required.
  - But it is difficult to analyze the result and it needs good high throughput screening system.

Approaches for enzyme stability

- **Rational design**
  - Rational design method is to redesign of protein based on the understanding of relationship between structure and function.
  - Rational design needs knowledge of the target protein's molecular structure.
  - Successfully established rational design method can be applied to increase stability of other proteins.

Approaches for enzyme stability

- **Computational protein design**
  - Computational protein design method is to investigate the sequence space of protein using scoring function and to find out most stable sequence of given protein backbone structure.
  - Computational protein design method is applied not only to protein stabilization but also to membrane protein solubilzation, novel enzyme design, and protein-protein interaction.



Target protein backbone

Searching sequence space by computational algorithm

Computationally designed protein

Case study 1: Protein Stabilization

(1) Structure–based pattern analysis for protein stability

(2) Increasing thermostability of Lipase A using rational design + computational design method

Understanding of protein thermostability

❍ **Factors affecting to stability of proteins :**

- Hydrophobic interaction
- Electrostatic interaction (e.g. salt bridge)
- Conformational flexibility
- Disulphide bond
- Hydrogen bond
- Aromatic interaction
- Metal binding
- …

⇨ **How to apply for the design?**

Comparative study for protein thermostability

- **Conventional approaches**

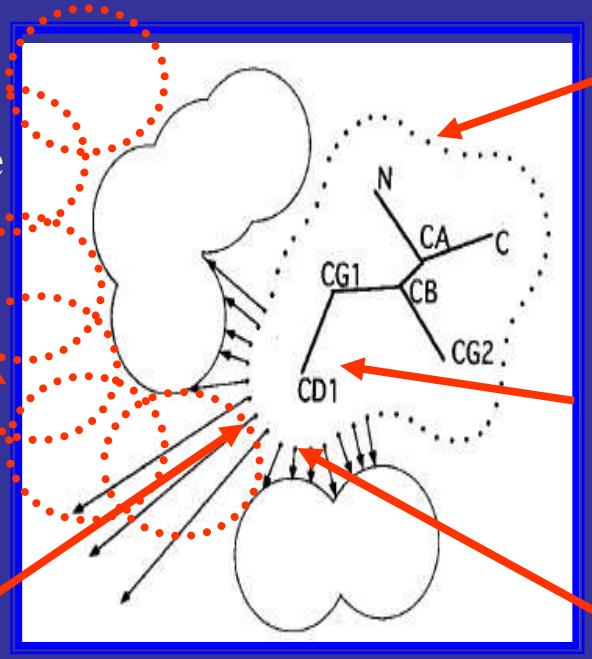
: Simple investigation such as one-dimensional difference of amino acid sequence, comparison of residual properties

- **Our approach :** Structure-based systematic analysis

: Investigation of the characteristic properties of model protein group in residual structure according to their conformational states

Development of rules & methods for thermostable protein design

Residual Packing Value



Water probe
(R=1.4 Å)

Molecular surface area (Ms_i)

Occluded surface area (OS_i)

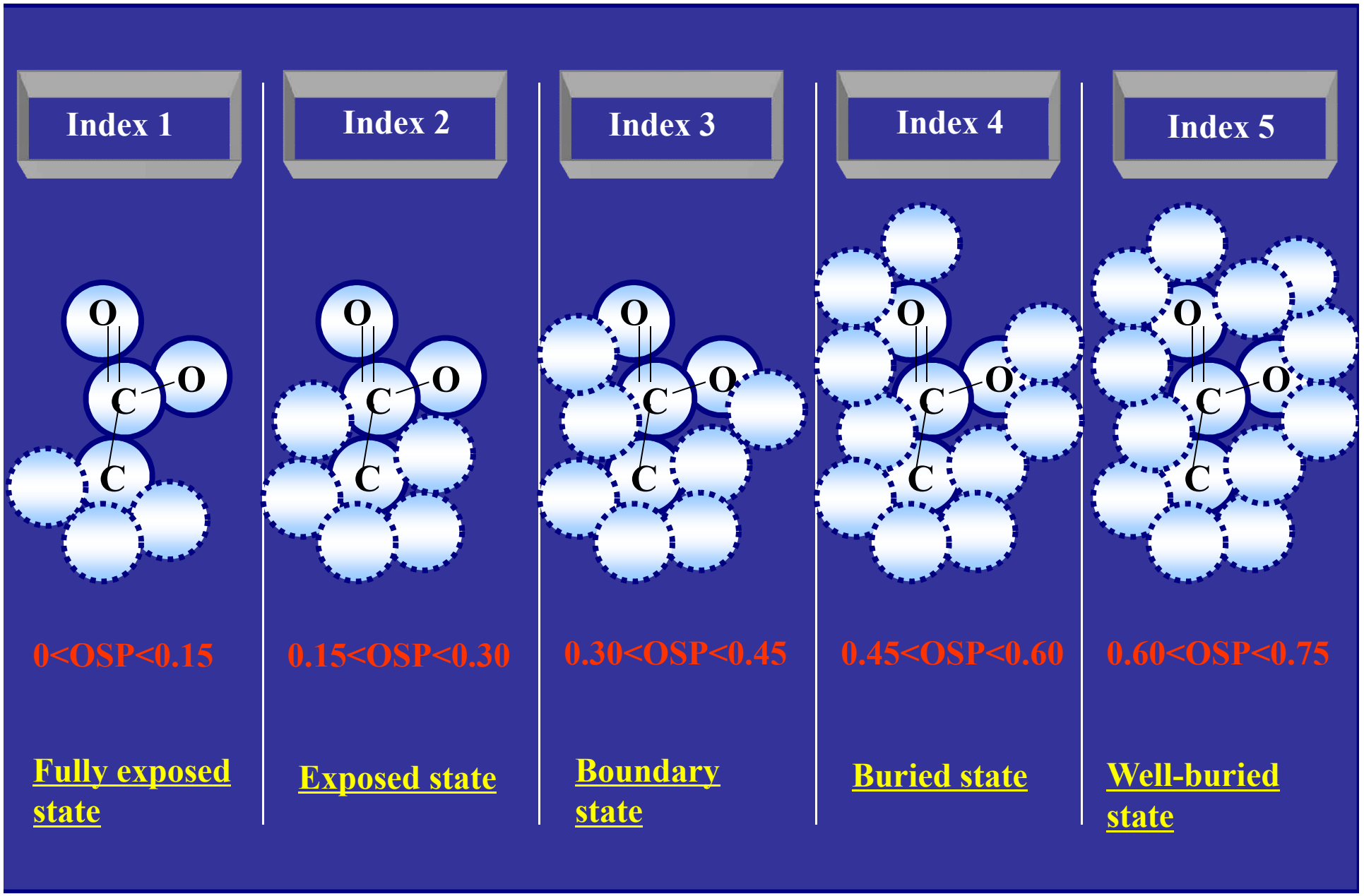
Exposed surface area (A_i^{ex})

Raylength (RL)

Residual packing value

$$OSP = \frac{(\Sigma(OS_i * \langle 1-RL \rangle_i))}{\Sigma\, MS_i}$$

Ref. : Fleming & Richard, 2000


Index 1
Index 2
Index 3
Index 4
Index 5
O
O
C
C
0<OSP<0.15
0.15<OSP<0.30
0.30<OSP<0.45
0.45<OSP<0.60
0.60<OSP<0.75
Fully exposed state
Exposed state
Boundary state
Buried state
Well-buried state

20 Set of Thermophilic and Mesophilic Proteins

	Thermophilic proteins			Mesophilic proteins		
Protein name	**PDB code**	**Organism**	**Temp**	**PDB code**	**Organism**	**Temp**
Adenylate kinase	1zin	Bacillus stearothermopilus	45-65	1aky	Sacchromyces cerevisae	25-30
Che Y	1tmy	Thermotoga maritima	80	3chy	Escherichia	37
Cirate synthase	1aj8	Pyrococcus furiosus	100	1csh	Chicken herat	37
Triose phosphate	1btm	Bacillus stearothermophilus	40-65	1ypi	Saccharomyces cerevisiae	25-30
Dimerization domain of EF-TS and EF-TU-TS complex	1tfe	Thermus thermophilus	70-75	1efu_b	Escherichia coli	37
Endo-1.4-b Xylanase	1yna	Thermomyces lanuginosus	50	1xnb	Bacillus circulans	30-40
Glutamate dehydrogenase	1gtm	Pyrcocus furiosus	75-100	1hrd	Clostridium symbiosum	30-37
Inorganic pyrophosphatase	2prd	Thermus thermophilus	70-75	1ino	Escherichia coli	37
Lactate dehydrogenase	1ldn	Bacillus stearothermophlic	40-65	1ldg	Plasmodium falciparum	37
Ribonuclease H	1ril	Thermus therimophilus	70-75	2rn2	E. coli	70-75

	Thermophilic proteins			Mesophilic proteins		
Protein name	**PDB code**	**Organism**	**Temp**	**PDB code**	**Organism**	**Temp**
Malate dehydrogenase	1bdm	Thermus flavus	70-75	4mdh	Porcine	37
Manganese superoxide	3mds	Thermus therimophilus	70-75	1qmn	Homo sapiens	37
Methionine aminopeptidase	1xgs	Pyrococcus furiosus	100	1mat	Escherichia coli	37
Phsophofructokinase	3pfk	Bacillus stearothermophilus	40-65	2pfk	Escherichia coli	37
3-Phosphoglycerate kinase	1php	Balcillus stearothermophilus	40-65	1qpg	Saccharomyces cerevisiae	25-30
Rubredoxin	1caa	Pyrococcus furiosus	100	8rxn	Desulfovibrio vulgaris	34-37
Thermolysin and neutral protease	1lnf	Bacillus thermoproteolyticus	52.5	1npc	Bacillus cereus	30
Glyceraldehyde-3-phosphate dehydrogenase	1hdg	Thermotoga maritima	80-85	1gad	Escherichia coli	37
Reductase	1ebd	Bacillus stearothermophilus	40-65	1lpf	Pseudomonas fluorescens	25-30
Subtilisin	1thm	Thermoactinomyces vulgaris	55-65	1st3	Bacillus lentus	30

Analyzed residual properties

- **Packing pattern**
- **Residual structural properties**

  : hydrogen bond, salt bridge, cation pi interaction, disulfide bond, inner, outer, flexible, rigid residue
- **Amino acid preference**

  : 20 amino acid
- **Secondary structure**

  : extended beta, beta strand, helix, 3/10 helix, turn

Statistical analysis of residual properties

T-test : Quantitative evaluation of difference between $X_{i\text{-}Th}$ and $X_{i\text{-}Me}$

t value … $t_i = (X_{i\text{-}Th} - X_{i\text{-}Me}) / \sqrt{(S^2_{i\text{-}Th}/N_{Th} + S^2_{i\text{-}Me}/N_{Me})}$

$df = N_{Th} + N_{Me} - 2 = 38$

Df	$t_{0.1}$	$t_{0.05}$	$t_{0.025}$	$t_{0.01}$	$t_{0.005}$
Inf (>30)	1.282	1.645	1.960	2.326	2.576

Under 10% level of significance ($t_{0.01} = 1.282$)

If t is over 1.282, the probability that $X_{i\text{-}Th}$ is greater than $X_{i\text{-}Me}$ is 90%.

If t is under –1.282, the probability that $X_{i\text{-}Th}$ is less than $X_{i\text{-}Me}$ is 90%.

Important Structural Patterns Related with Thermostability

Frequency

Structure index	Packing				
	Thermo		Meso		T-test
1	3.9948	±0.0869	4.6387	±0.0871	-1.1698
2	24.4497	±0.2211	25.9943	±0.2779	-0.9725
3	34.2689	±0.1499	33.6561	±0.1613	0.6224
4	32.9906	±0.2891	32.5293	±0.2720	0.2599
5	4.2959	±0.1297	3.1816	±0.0934	1.5586

Thermophilic protein

… higher frequency of residues in well-buried state

※ Guideline : more packing in well-buried state location

Important Structural Patterns Related with Thermostability

[Residual structural properties]

Characteristics	Location
1. Higher frequency of salt-bridge	Exposed state (index2)
2. Lower frequency of flexible residue	Fully-exposed state (index1)
3. Higher frequency of flexible residue	Boundary state (index3)
4. Higher frequency of hydrogen bonds	Well-buried state (index5)
5. Higher frequency of inner residue	Well-buried state (index5)

※ Guideline : ex) more salt bridges at exposed state location

Important Structural Patterns Related with Thermostability

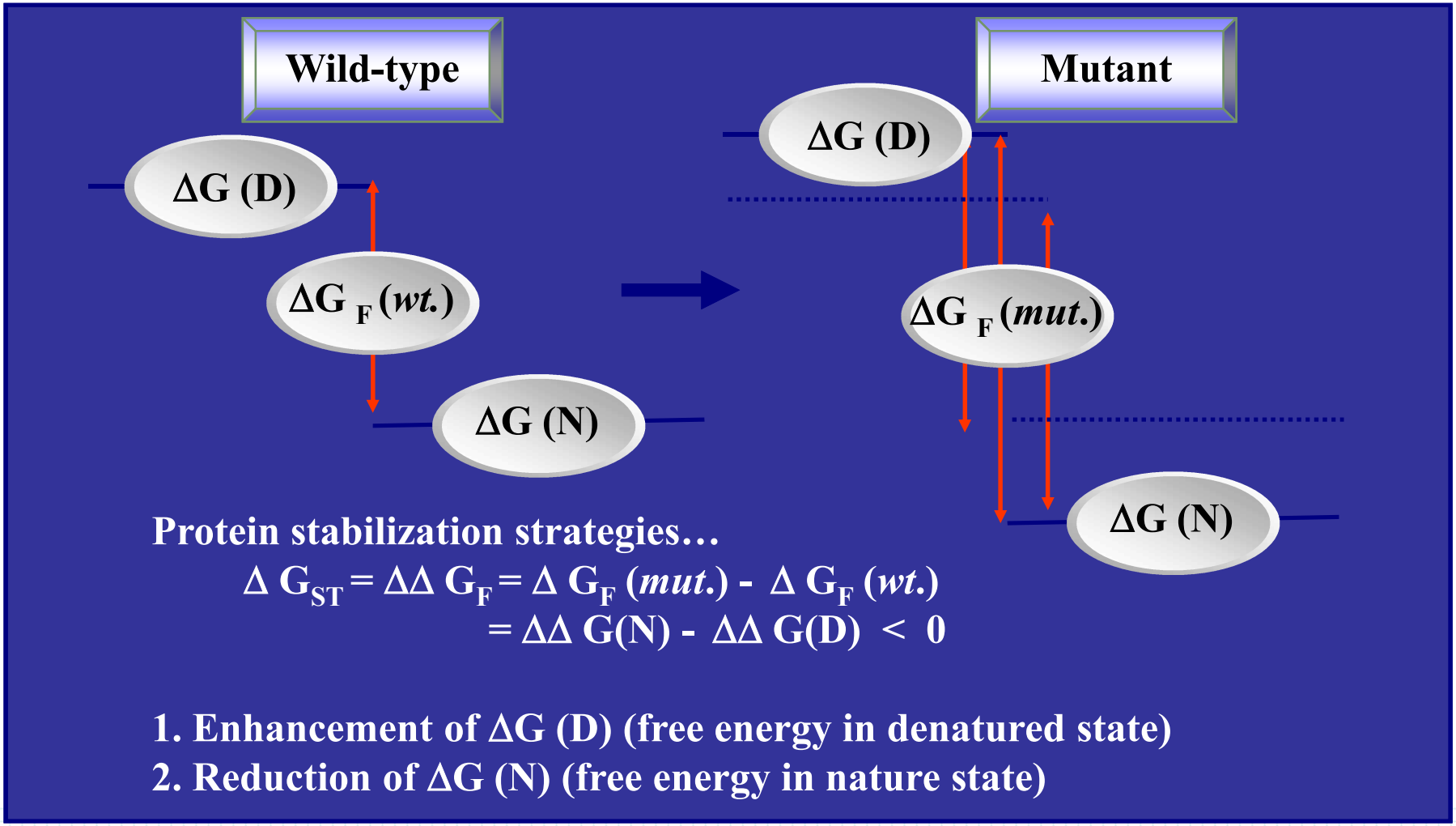
[Amino acid preference]

Characteristics	Location
1. Lower frequency of SER	Boundary state (index 3)
2. Lower frequency of ALA	Exposed state (index2)
3. Higher frequency of ALA	Well-buried state (index5)
4. Higher frequency of GLU	Buried state (index4)
5. Higher frequency of ARG	Exposed state (index2)

Case study (II)

- Increasing thermostability of Lip A using rational + computational design method

Criteria for Stable Protein

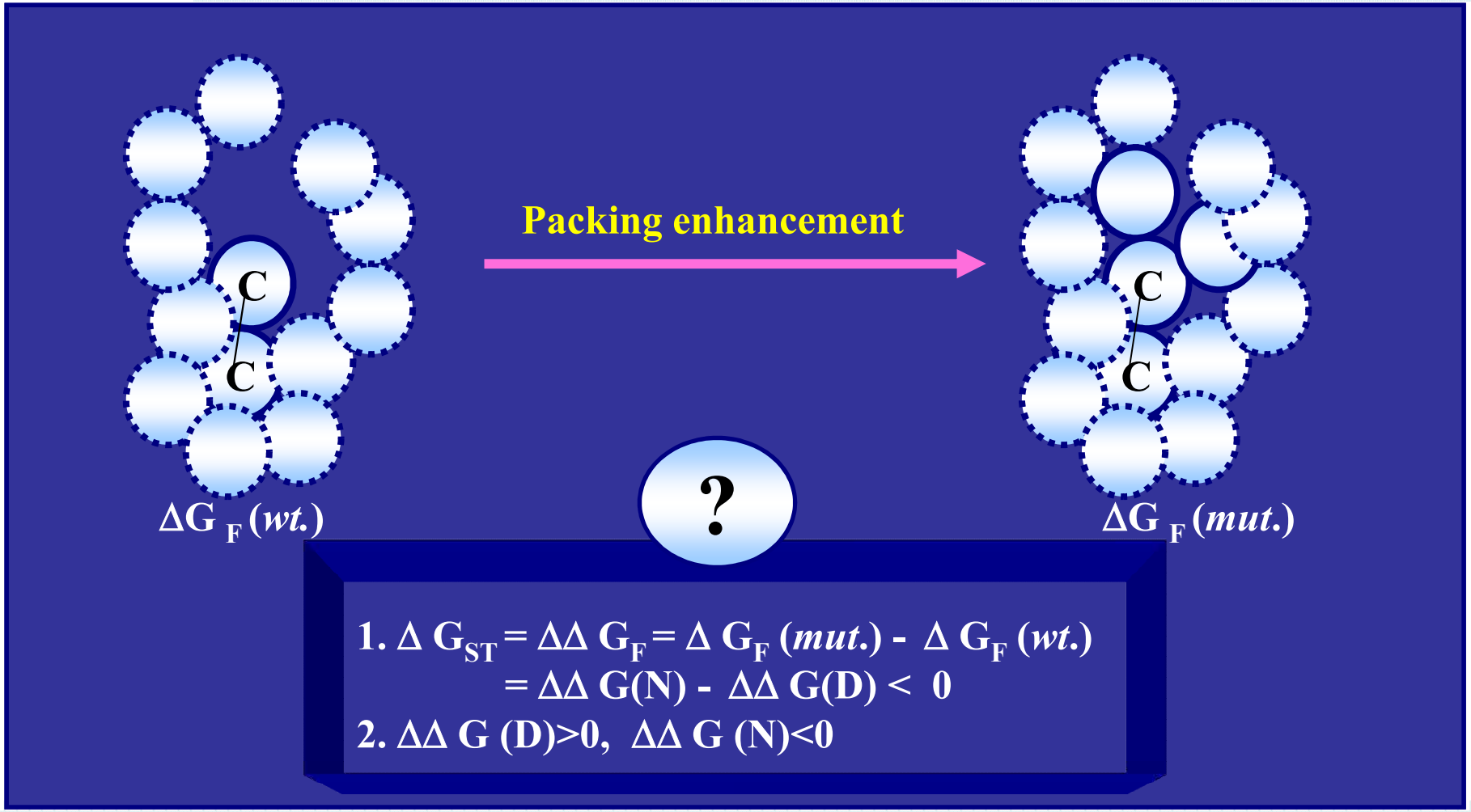


Protein stabilization strategies…

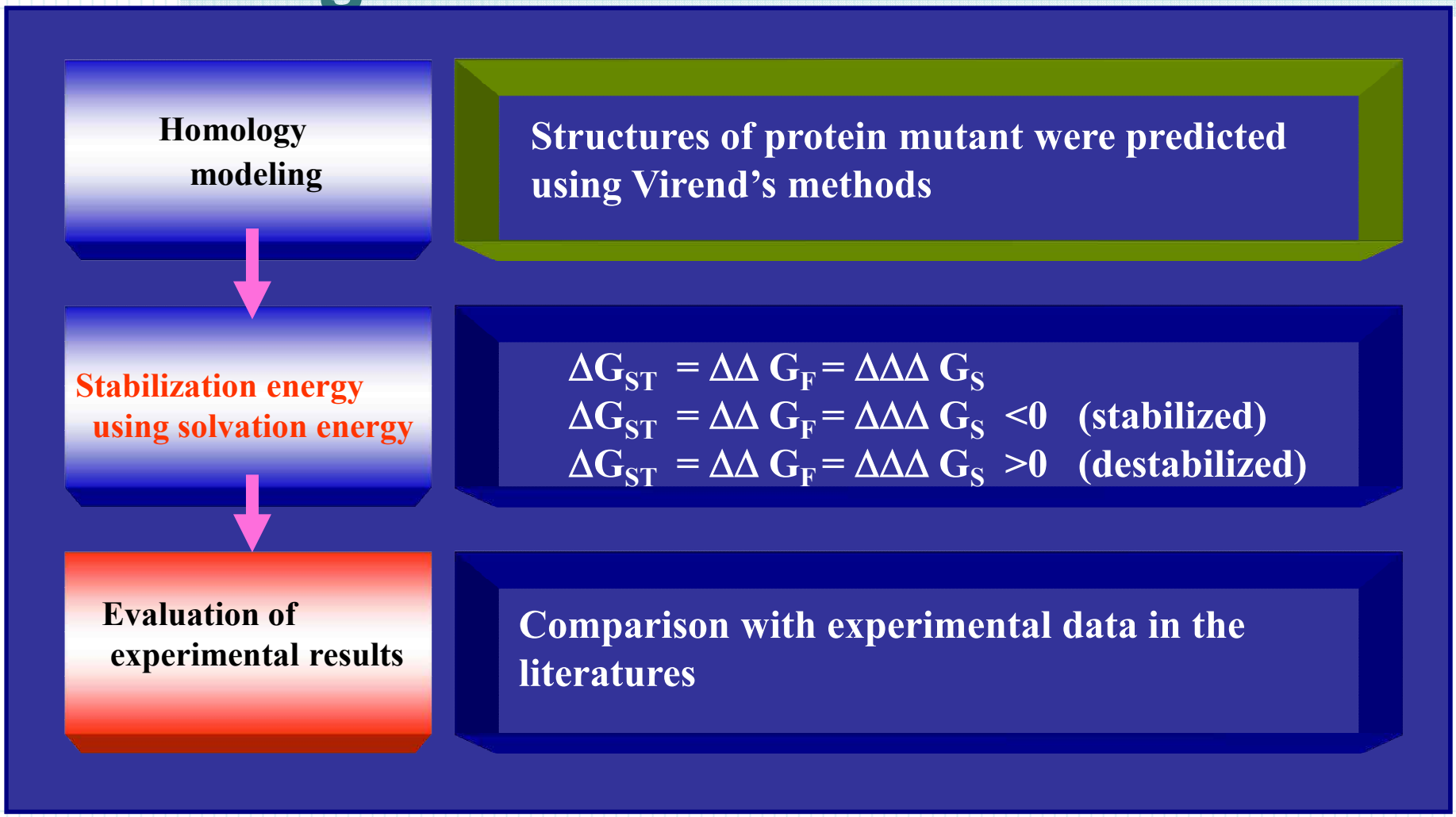
$$\Delta G_{ST} = \Delta\Delta G_F = \Delta G_F\,(mut.) - \Delta G_F\,(wt.)$$
$$= \Delta\Delta G(N) - \Delta\Delta G(D) < 0$$

1. Enhancement of ΔG (D) (free energy in denatured state)
2. Reduction of ΔG (N) (free energy in nature state)

Packing Enhancement for Protein Stability



Prediction and Evaluation of Packing Effect



Model Proteins

PDB ID	2LZM	1STN	2RN2	4LYZ	2WSY	1BNI
Name	**Lysozyme**	**Staphylococcal Nuclease**	**Ribonuclease HI**	**Lysozyme**	**Tryptophan Synthase**	**Barnase**
EC number	3.2.1.17	3.1.31.3	3.1.26.4	3.2.1.17	4.2.1.20	3.1.27.-
Family	Hydrolase (o-glycosyl)	Hydrolase (phosphoric diester)	Hydrolase (endoribonuclease)	Hydrolase (o-glycosyl)	Lyase	Microbial ribonuclease
Source	*Bacteriophage T4*	*Staphylococcus aureus*	*Escherichia coli*	*Hen egg white*	*Salmonella typhimurium*	*Bacillus amyloliquefaciens*
Resoultion	1.73	1.70	1.48	2.00	2.30	2.10
R-factor	0.193	0.162	0.196	Not reported	0.197	0.179

Mutants	**G77A**	**G88A**	**G23A,A52N A52D,A52Q A52E,A52I A52L,A52V**	**A31I A31L A31V**	**A18V**	**I51A, I51V I76A, I76V V10A**

Structural Properties of Target Residues

Target residues are inner residues (below 5% exposure ratio)
Their structural states are the buried-state (below 0.65 packing value)

Model Protein	Residues	Exposure ratio (%)	Packing value	No. of methyl or methylene
2LZM	GLY77	4.5	0.550	0
1STN	GLY88	0.1	0.554	0
2RN2	GLY23	0.0	0.597	0
	ALA52	0.5	0.482	1
4LYZ	ALA31	0.0	0.486	1
2WSY	ALA18	0.5	0.525	1
1BNI	VAL10	0.0	0.616	3
	ILE51	0.1	0.519	4
	ILE76	0.0	0.641	4

For prediction of stabilization effect

For prediction of destabilization effect

Stabilization Effect of Packing Enhancement

Comparison of predicted and experimental $\Delta\Delta G_F$ of mutant variants

Mutant		Prediction ($\Delta\Delta G_F$)				Experiments ($\Delta\Delta G_F$)	
		Em86	Sch1	Sch2	This work	$\Delta\Delta G_F$	ΔTm
2LZM	G77A	-1.29	-1.37	-1.05	-2.20	-0.40	0.90
1STN	G88V	-3.60	-3.85	-3.35	-3.62	-0.60	-
2RN2	G23A	-1.83	-1.97	-1.72	-2.36	-0.70	2.3

$$\Delta G_{ST} = \Delta\Delta G_F = \Delta G_F\,(mut.) - \Delta G_F\,(wt.) < 0$$

Stabilization effect of GLY to ALA or VAL could be explained

Comparison of predicted and experimental ΔΔ G_F of mutant variants

Mutant		Prediction ($\Delta\Delta G_F$)				Experiments ($\Delta\Delta G_F$)	
		Em86	Sch1	Sch2	This work	$\Delta\Delta G_F$	ΔTm
2RN2	A52N	0.999	0.820	1.211	1.047	1.80	-5.90
	A52D	1.482	2.031	5.197	1.571	1.90	-6.10
	A52Q	0.607	0.361	0.880	0.710	1.20	-3.90
	A52E	1.090	1.629	5.163	1.249	1.50	-5.00
	A52I	-1.471	-1.736	-0.656	-1.901	-1.90	6.20
	A52L	-1.513	-1.790	-0.695	-1.972	-1.30	4.30
	A52V	-1.043	-1.259	-0.311	-1.547	-1.70	5.50
4LYZ	A31I	-2.37	-2.60	-2.17	-2.25	-1.4	3.6
	A31L	-2.42	-2.66	-2.21	-2.31	-1.8	4.7
	A31V	-1.95	-2.13	-1.83	-1.91	-1.2	3.1
2WSY	A18V	-0.76	-0.80	-0.75	-0.88	-0.8	-

Stabilization effect of ALA to ILE, LEU or VAL could be explained

Destabilization Effect of Packing Decrease

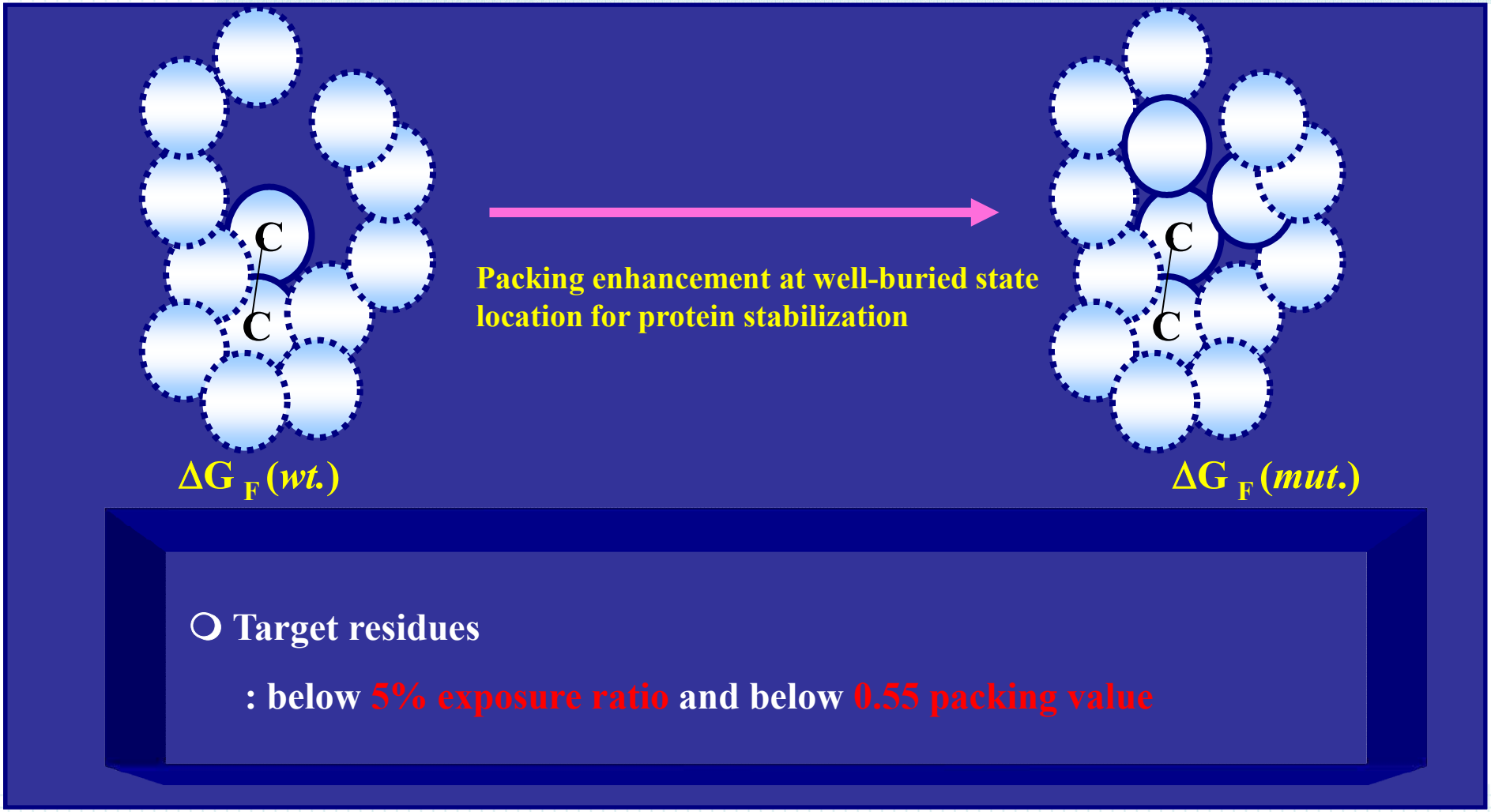
Comparison of predicted and experimental $\Delta\Delta G_F$ of mutant variants

	Prediction ($\Delta\Delta G_F$)				Experiments ($\Delta\Delta G_F$)
Mutant	Em86	Sch1	Sch2	This work	$\Delta\Delta G_F$
I51A	1.58	1.67	1.30	1.95	4.71
I51V	0.77	0.73	0.66	1.23	1.80
I76A	1.57	1.65	1.32	2.19	1.89
I76V	0.77	0.73	0.66	1.23	0.82
V10A	1.15	1.18	0.98	1.71	3.39

$$\Delta G_{ST} = \Delta\Delta G_F = \Delta G_F\,(mut.) - \Delta G_F\,(wt.) > 0$$

Destabilization effect of ILE to ALA or VAL could be explained

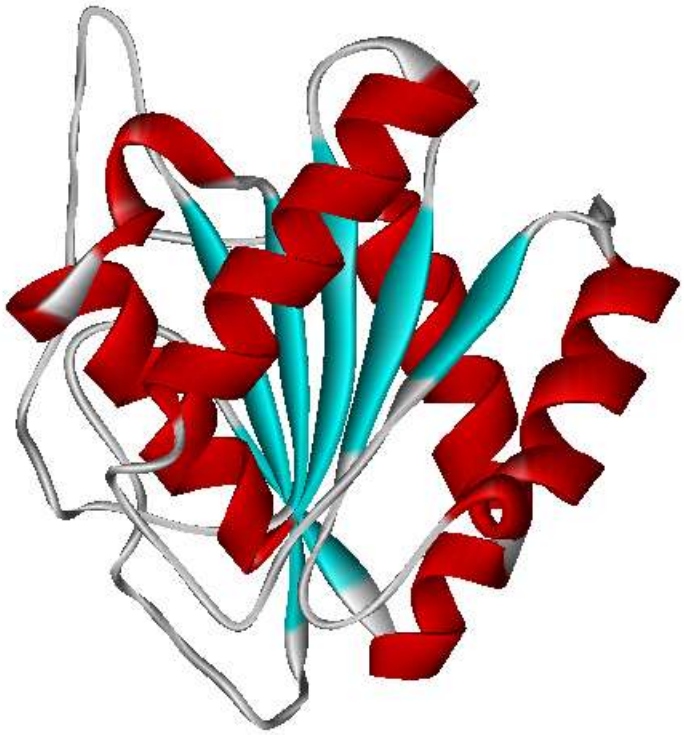
Proposed Stabilized Strategy



- Target residues

  : below 5% exposure ratio and below 0.55 packing value

Model Enzyme



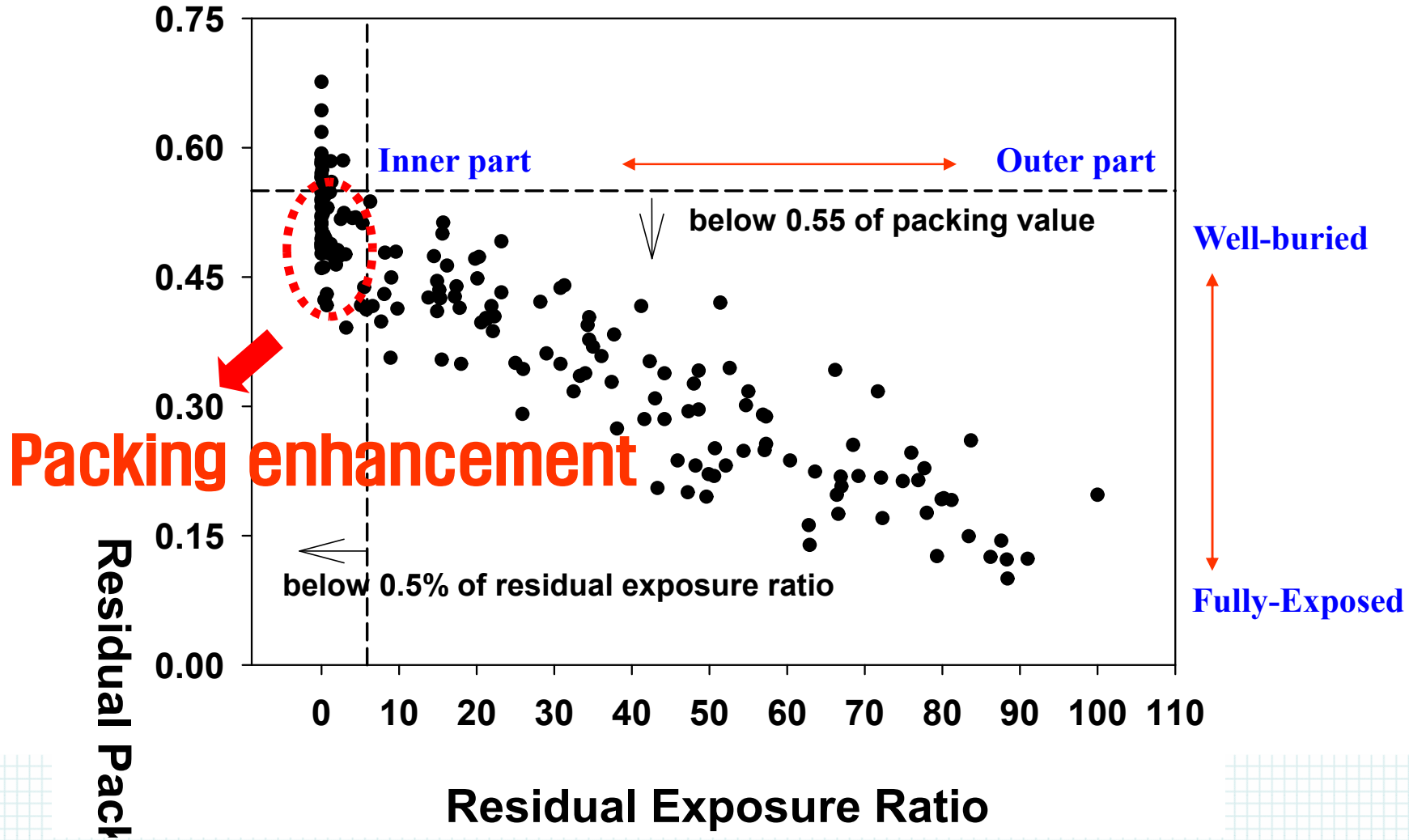
Lipase A from *Bacillus subtilis*

- Alkaline pH optima (10.0)
- Small size … 179 a.a. , 19.3 kDa
- No lid
- Optimum Temp … 35-40^0 C
- PDB code : 1i6w

Stabilization of Lipase A

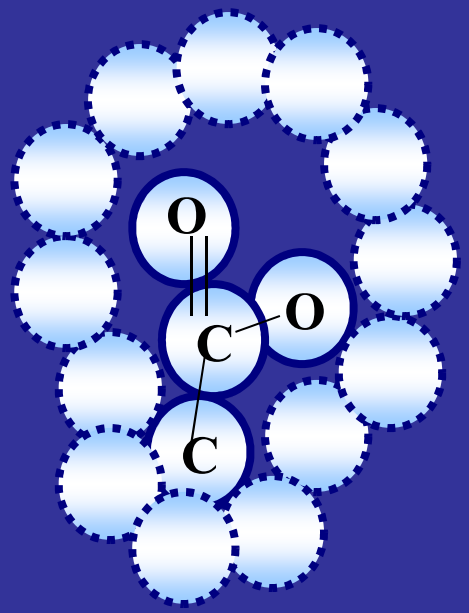
- **Rational + computational design of Lipase A**
  - The introduction of well-packed residues to inside of the protein could be considered as one of the stabilization strategies.
  - How to design the inner packing of protein structure for protein thermostabilization?

Selection of Target Residues Based on Residual Packing Value and Exposure Ratio



Residues Which Need to Have More Packing

1st Criteria … below 5% exposure ratio and below 0.55 packing value



43 residues were selected

PRO	5	VAL	74	THR	109
VAL	6	ALA	75	THR	126
VAL	7	HIS	76	SER	127
HIS	10	GLY	80	ILE	128
PHE	19	ASN	82	ASP	133
ILE	22	THR	83	SER	141
TRP	31	ILE	87	LEU	143
LEU	36	VAL	96	ALA	146
ALA	38	VAL	99	ILE	151
VAL	39	VAL	100	LEU	159
PHE	41	THR	101	LEU	160
VAL	62	LEU	102	ILE	169
ASP	72	GLY	103	GLY	172
ILE	73	ALA	105	LEU	173
				ASN	179

2nd criteria … • **Among 43 residues, GLY and ALA were considered .**
(In terms of packing enhancement, small amino acid would be proper as target residues.)

• **Gly to ALA, ALA to ILE, LEU or VAL**

ALA

Amino acid	Number	Ratio	Packing
ALA	38	1.9	0.464
ALA	75	0	0.521
ALA	105	2.5	0.517
ALA	146	0	0.486

ALA to VAL

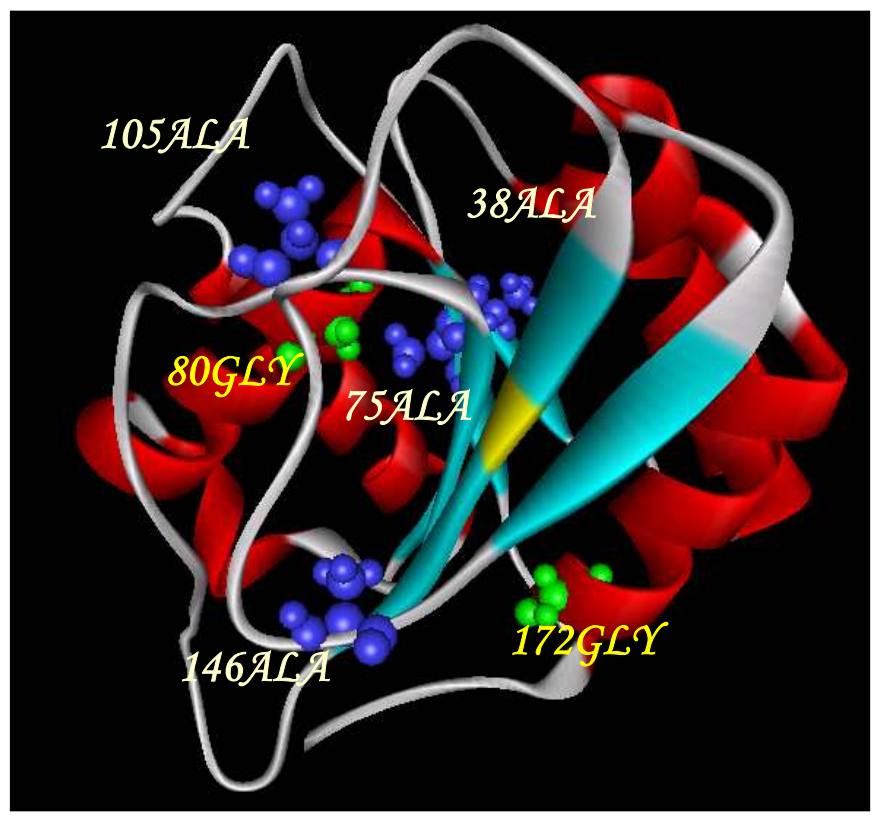
GLY

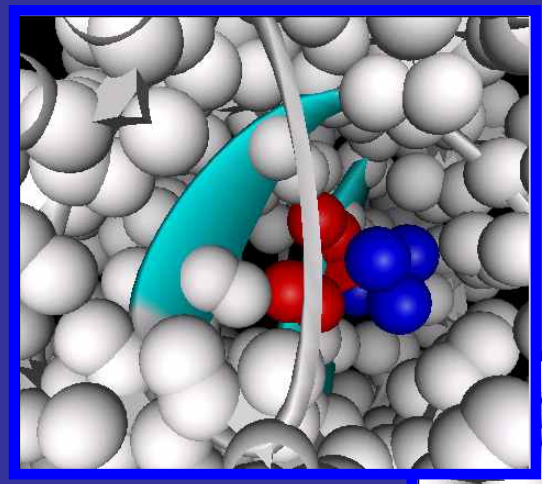
Amino acid	Number	Ratio	Packing
GLY	80	0	0.548
GLY	103	0.5	0.495
GLY	172	1	0.477

GLY to ALA

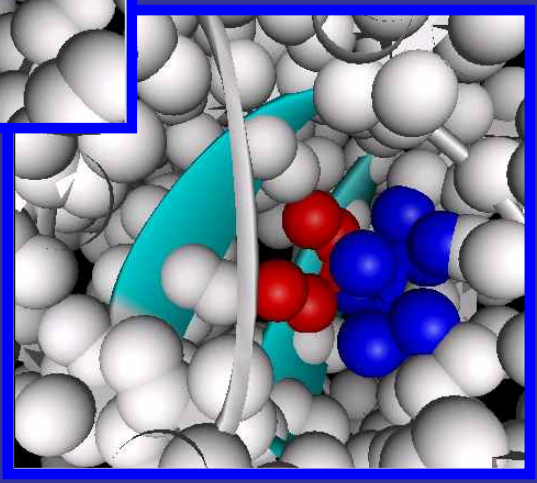
Selected Residues

(Ex) 75 residue



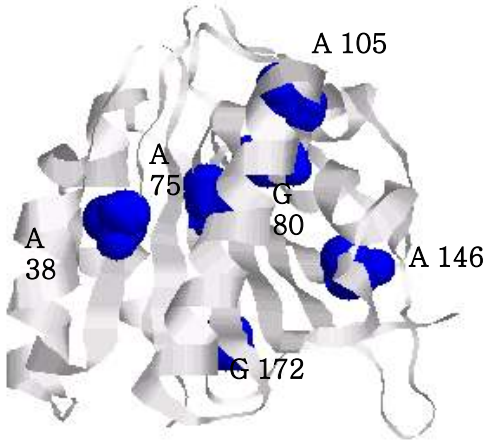


Ala → Val

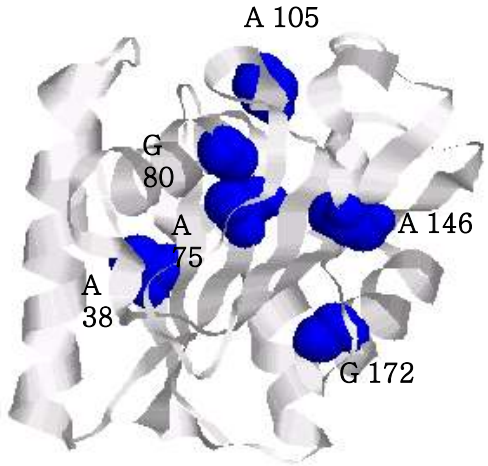


Residual packing
Enhancement !!!

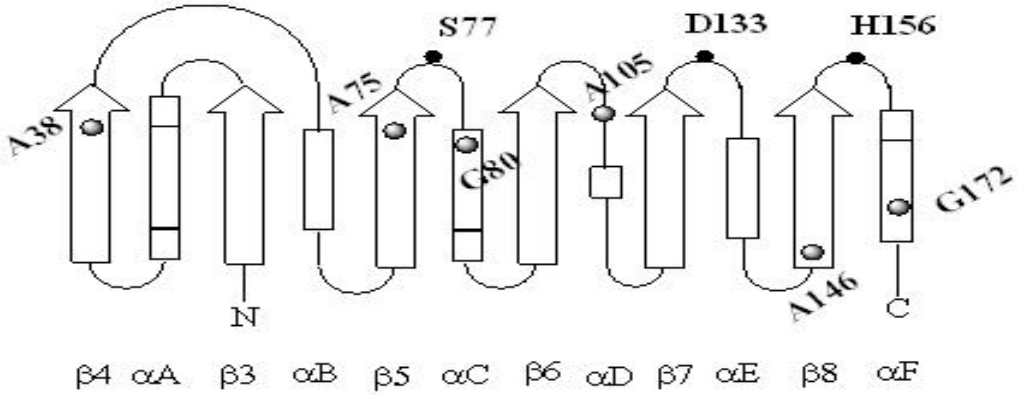
Structural View of Mutational Target Sites in B. subtilis Lipase A

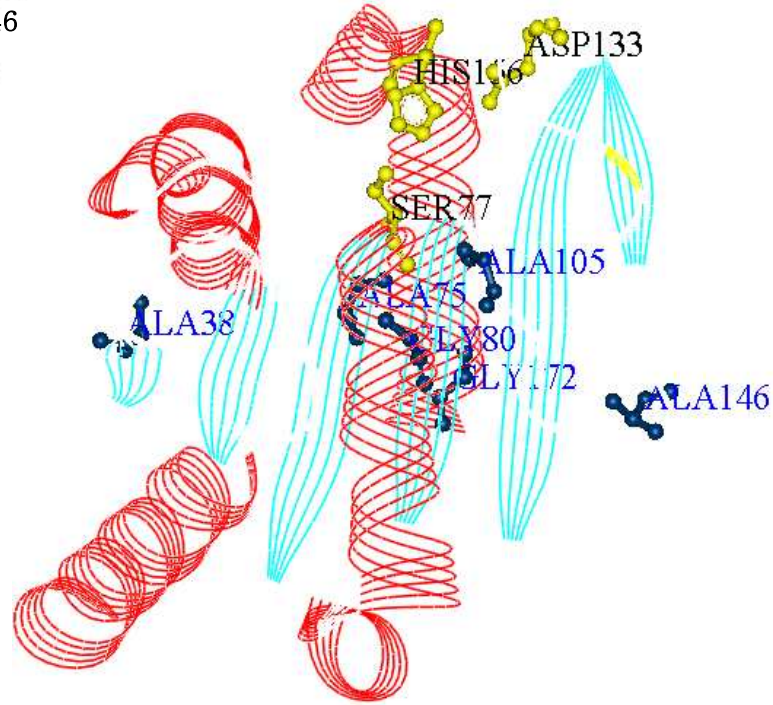


(A) One of view of 1i6w



(B) Rotational view





(C) Location of target sites on secondary structure and tertiary structure

Prediction of Packing Effect to Conformational Stability of Mutant Proteins

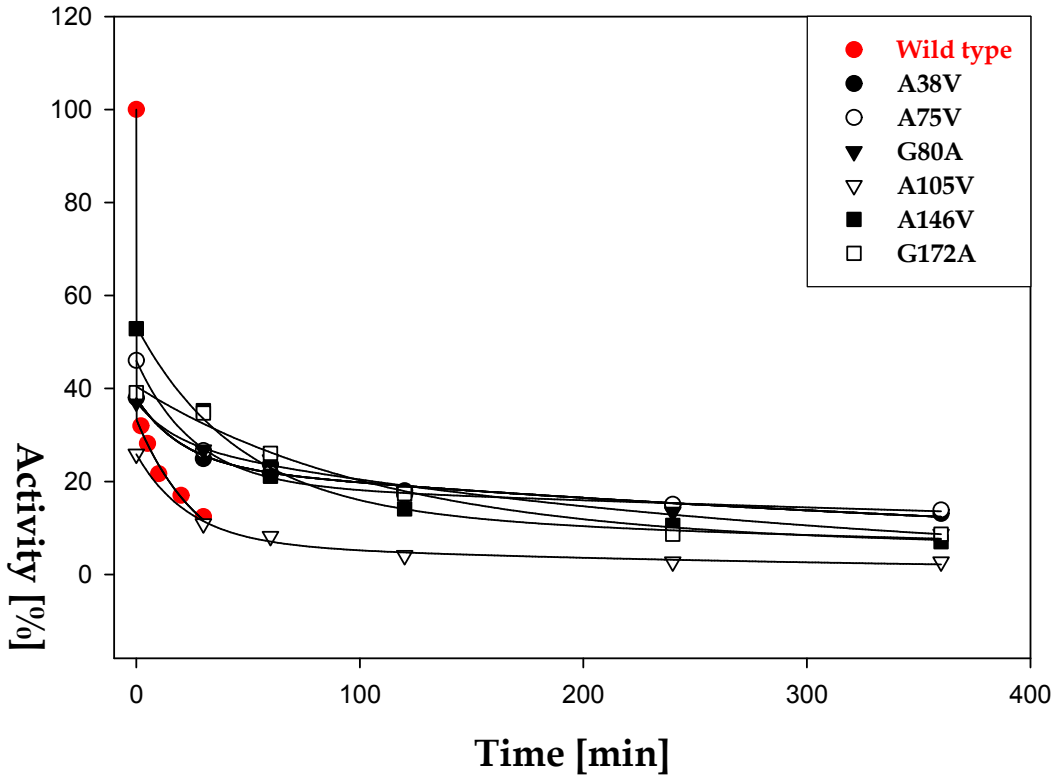
Mutant	$\Delta\Delta\Delta G_f$
A38V	-0.7433
A75V	-0.8076
A105V	-0.8869
A146V	-0.8736
G80A	-0.5368
G172A	-0.5553

Through homology modeling and packing-considered investigation, 0.5 – 1.0 kcal/mol stabilization effect of packing was predicted.

The Kinetic Parameter, Specific Activity and Thermostability (half-life (t1/2) in 50 ^{0}C , pH 5.5)

	Specific activity (U mg^{-1})	$t_{1/2}$ (min)
wild-type	3360.0	1.6
A38V	1178.0	107.5
A75V	195.5	47.5
G80A	985.8	113.8
A105V	857.4	26.5
A146V	950.0	48.5
G172A	863.4	102.5

Thermostability of Mutant at 50.0 ^{0}C (pH 5.5)



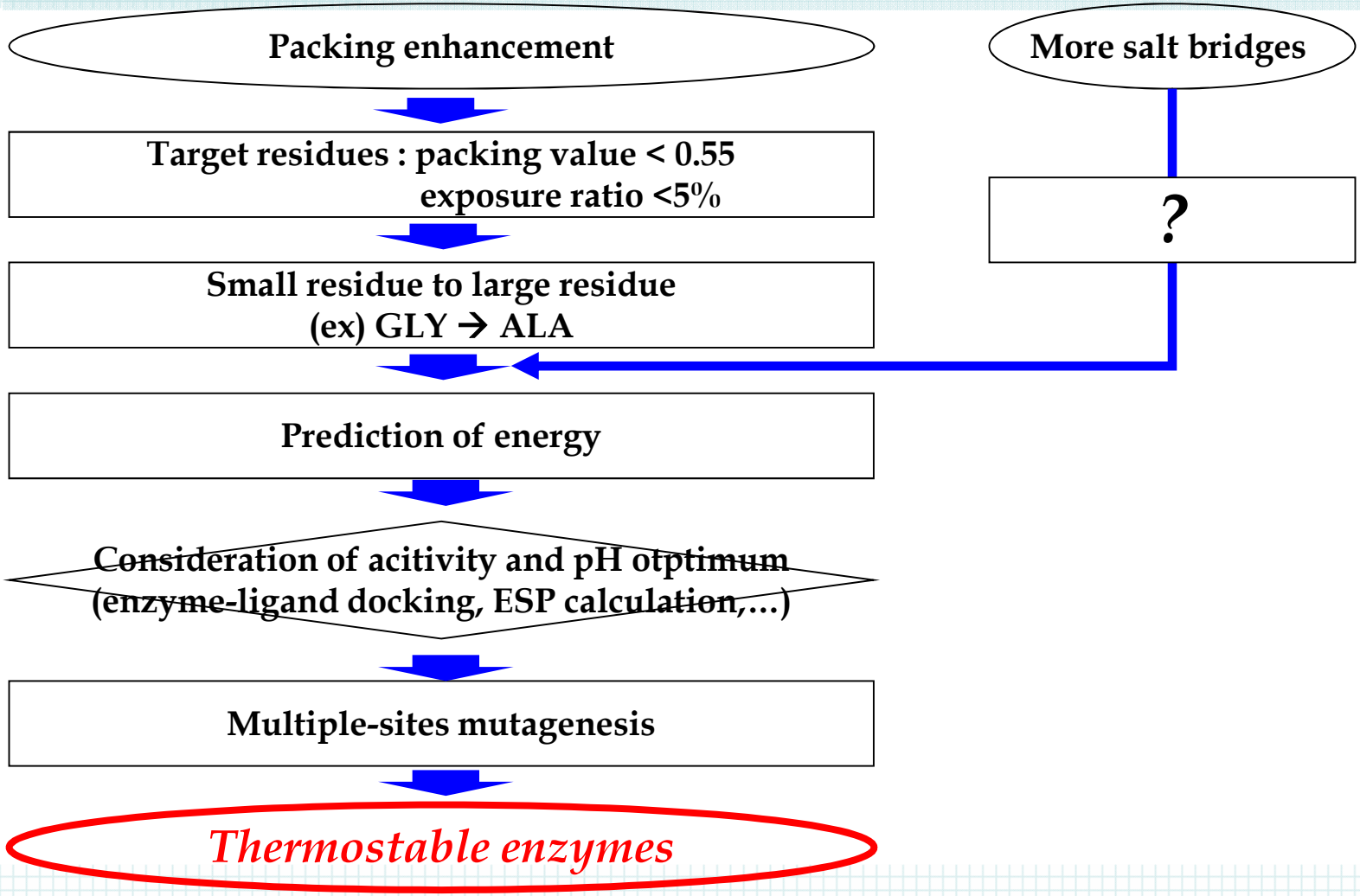
The thermostability assay revealed that the A38V , G80A and G172V are promising among the mutants

Conformational Stability of Multiple-Site Mutants

Single mutant	ΔΔΔ Gf
A38V	-0.7433
A75V	-0.8076
A105V	-0.8869
A146V	-0.8736
G80A	-0.5368
G172A	-0.5553

Double mutant	ΔΔΔ Gf
A38V_A75V	-1.5509
A38V_A105V	-1.6302
A38V_A146V	-1.6169
A38V_G80A	-1.2873
A38V_G172A	-1.2986
A75V_A105V	-1.6945
A75V_A146V	-1.6811
A75V_G80A	-1.354
A75V_G172A	-1.3629
A105V_A146V	-1.7605
A105V_G80A	-1.4229
A105V_G172A	-1.4422
A146V_G80A	-1.4104
A146V_G172A	-1.4289
G80A_G172A	-1.0921

Computational and Rational Design of Enzyme Thermostability



Critical Thinking

1. Think about the relationship between thermodynamic stability and kinetic stability
2. Search the difference between urea and guanidinium on enzyme unfolding

유기용매 안정성

메탄올과 같은 유기용매 하에서는 효소가 활성을 잃는다.

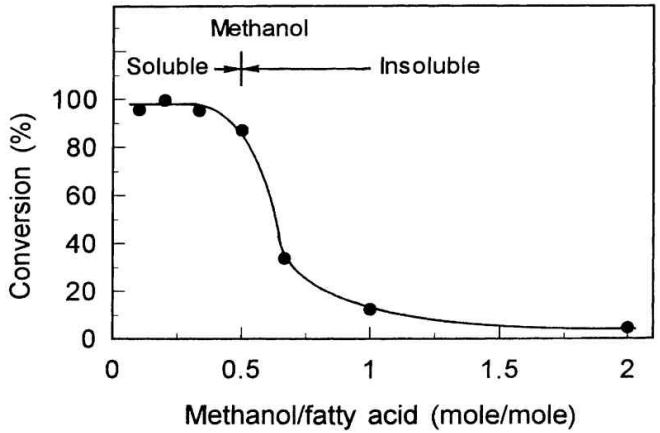
- Literature survey
  - The decrement of conversion rate by adding > ½ molar equivalent methanol

    Yuji Shimada, Yomi Watanabe, Akio Sugihara, and Yoshio Tominaga, Enzymatic alcoholysis for biodiesel fuel production and application of the reaction to oil processing, Journal of Molecular Catalysis B: Enzymatic 17 (2002) 133–142

  - CalB deactivation by contact with methanol in hydrophobic oil

  - No significant change of CalB structure in hydrophilic organic solvent like methanol

    Peter Trodler and Jürgen Pleiss, Modeling structure and flexibility of *Candida antarctica* lipase B in organic solvents, BMC Structural Biology 2008, 8:9 doi:10.1186/1472-6807-8-9



Methanolysis of vegetable oil in various methanol/fatty acid molar ratio

일반적인 효소개량 방법

- - 유기용매 (solvent) engineering
- - Directed evolution (random mutation)
- - Rational approach

서울대의 효소개량 방법

- **Computational approaches :** 분자 모델링에 기반을 둔 이론적이고 논리적인 새로운 방법

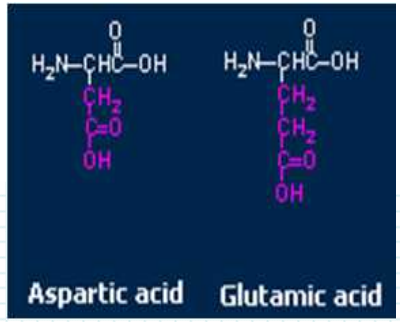
Computational approaches for biocatalyst improvement : 3가지 방법 –세계최초의 독창적 방법

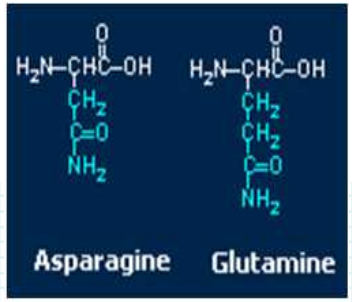
방법 I	• 수소결합 도입과 강화를 이용한 **CalB**의 메탄올 안정성 향상
방법II	• **유기용매 접촉부분 예측 및 유기용매 접촉을 못하게 함으로서 CalB의 메탄올 안정성 향상**
방법III	• **CalB**의 **flexbility** 증대를 통한 활성 증가

Strengthening of hydrogen bond network

방법 **1.** 수소결합 도입과 강화를 이용한 **CalB**의 메탄올 안정성 향상 연구

- Objective
  - Enhancing of methanol tolerance by introduction and strengthening of hydrogen bonding network between enzyme and water molecules in the hydration shell
- Methods
  - Selection of ASP and ASN at the loop to minimize 3D structure change of CalB
  - *in silico* mutations of ASP → GLU, ASN → GLN
  - Calculation of number and length of hydrogen bonding
  - Calculation of solvent accessible surface area of mutated sites
  - HBAT (Hydrogen Bond Analysis Tool)
  - Calculation of Aromatic-aromatic, Aromatic-sulphur interaction, Ionic interaction, cation-pi interaction.





Strengthening of hydrogen bond network

WT, D223E,N97Q,N196Q,N206Q,N264Q,N292Q
Ionic Interactions within 6 Angstroms

Position	Residue	Chain	Position	Residue	Chain
13	LYS	A	17	ASP	A
126	ASP	A	127	ARG	A
187	ASP	A	224	HIS	A
238	ARG	A	257	ASP	A
238	ARG	A	265	ASP	A
249	ARG	A	252	ASP	A
294	GLU	A	308	LYS	A
296	ASP	A	302	ARG	A

D265E
Ionic Interactions within 6 Angstroms

Position	Residue	Chain	Position	Residue	Chain
13	LYS	A	17	ASP	A
126	ASP	A	127	ARG	A
187	ASP	A	224	HIS	A
238	ARG	A	257	ASP	A
238	ARG	A	265	GLU	A
249	ARG	A	252	ASP	A
294	GLU	A	308	LYS	A
296	ASP	A	302	ARG	A

N96Q
Ionic Interactions within 6 Angstroms

Position	Residue	Chain	Position	Residue	Chain
13	LYS	A	17	ASP	A
98	LYS	A	126	ASP	A
126	ASP	A	127	ARG	A
187	ASP	A	224	HIS	A
238	ARG	A	257	ASP	A
238	ARG	A	265	ASP	A
249	ARG	A	252	ASP	A
294	GLU	A	308	LYS	A
296	GLU	A	302	ARG	A

D296E
Ionic Interactions within 6 Angstroms

Position	Residue	Chain	Position	Residue	Chain
13	LYS	A	17	ASP	A
126	ASP	A	127	ARG	A
187	ASP	A	224	HIS	A
238	ARG	A	257	ASP	A
238	ARG	A	265	ASP	A
249	ARG	A	252	ASP	A
294	GLU	A	308	LYS	A
296	GLU	A	302	ARG	A

Strengthening of hydrogen bond network

Wild type

D223E,N97Q,N206Q,N264Q,N292Q
Cation-pi Interactions within 6 Angstroms

Position	Residue	Chain	Position	Residue	Chain
61	TYR	A	32	LYS	A
91	TYR	A	124	LYS	A
234	TYR	A	238	ARG	A
253	TYR	A	208	LYS	A

N196Q
Cation-pi Interactions within 6 Angstroms

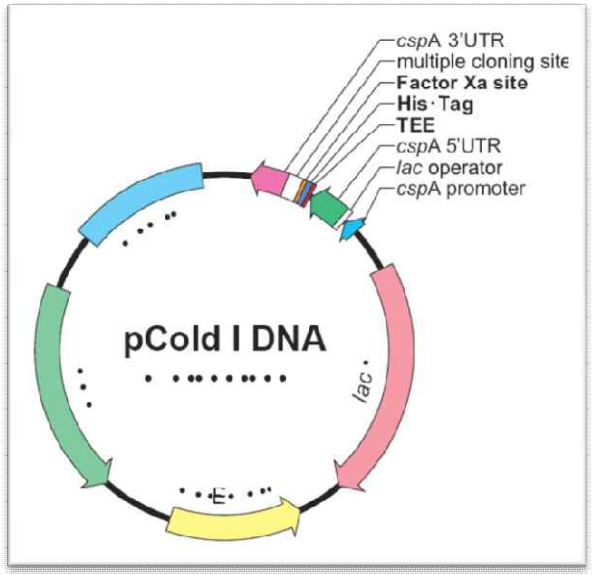
Position	Residue	Chain	Position	Residue	Chain
61	TYR	A	32	LYS	A
91	TYR	A	124	LYS	A
234	TYR	A	238	ARG	A

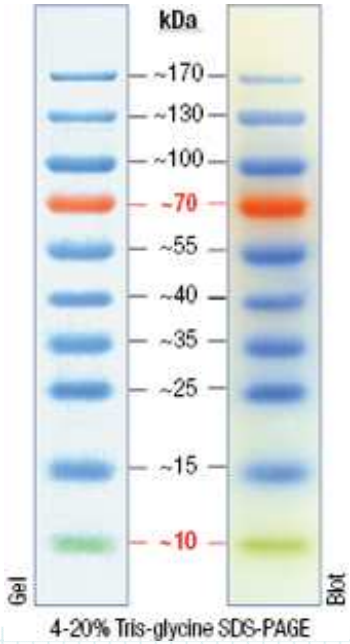
- D223E,D265E,D296E,N97Q,N206Q,N264Q,N292Q showed same intra molecule interaction
- N96Q showed additional ionic interaction.
- N196Q showed missing cation-pi interaction.
  (deselect for *in vitro* mutation)

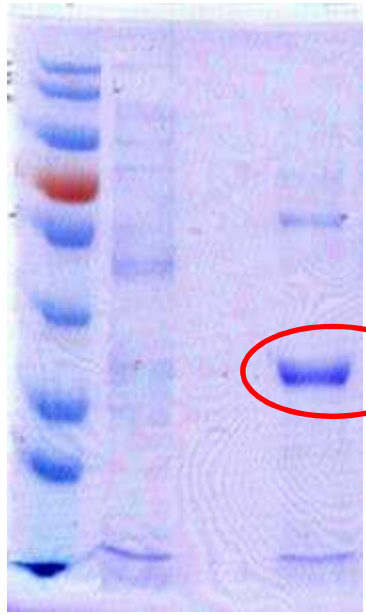
- Results
  - D223E, D265E , D296E, N96Q, N97Q, N206Q, N264Q, N292Q

In vitro Experiment

- Protein screening system
  - Vector : pColdI
    - Cold shock promoter, N-terminal 6-His tag
  - Cell : Origami 2(DE3)
  - Expression condition : 15˚C, 200rpm, 24hr

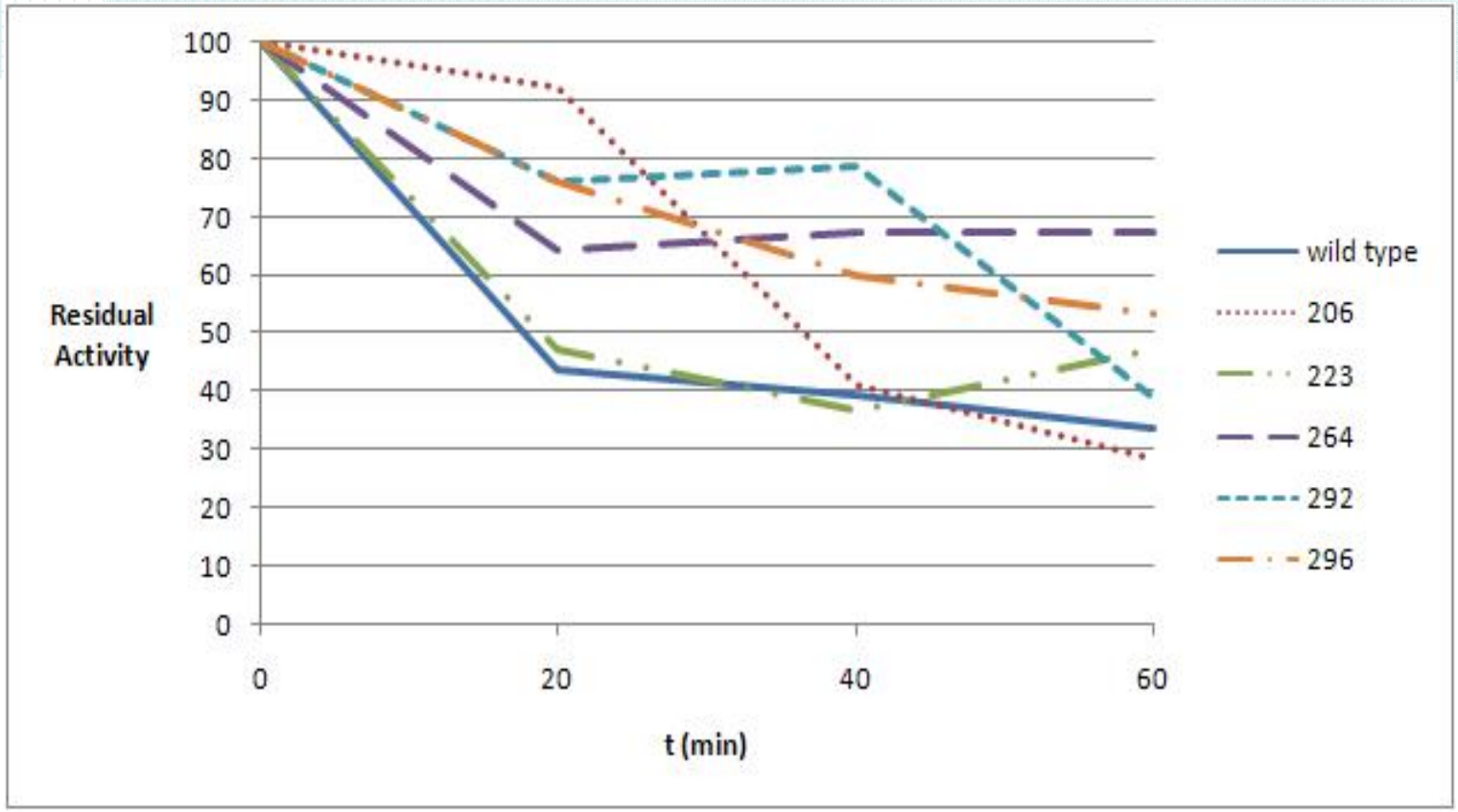






Soluble CalB

In vitro Experiment Results



- ❖ **D223E, D296E, N264Q, N292Q are more stable in methanol.**
- ❖ **Multiple site mutation** 실험 예정.
- ❖ 실제 **FAME**에 대한 실험 필요.

Predicting methanol binding sites

방법 2. 유기용매 접촉부분 예측 및 유기용매 접촉을 못하게 함으로서 CalB의 메탄올 안정성 향상 연구

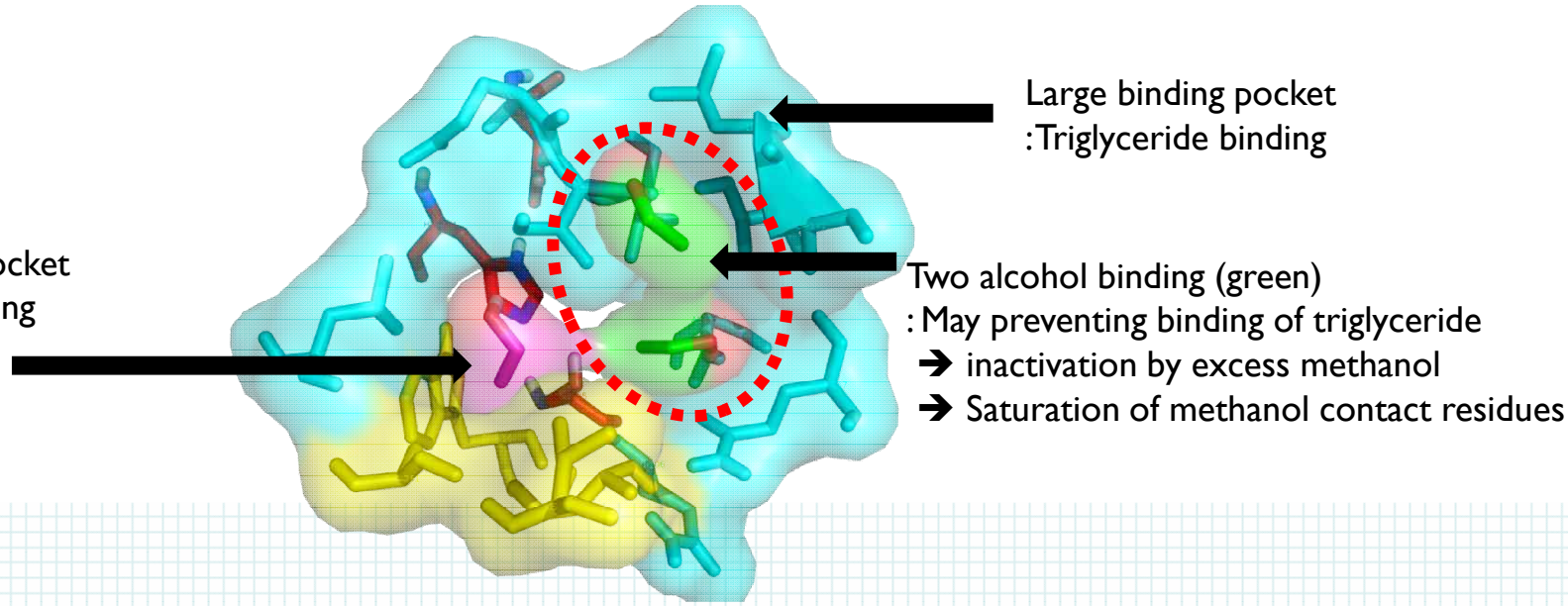
- CalB has small and large binding pockets in active site
- Small pocket for methanol binding as substrates and large pocket for triglyceride binding
- Prevention of methanol binding to large binding pocket may reduce the inactivation by excess methanol(>1/2 molar equivalent methanol)

- Methods
  - Methanol binding site prediction by FT-map server (http://ftmap.bu.edu/)
  - Alcohol probe: Ethanol, isobutanol and isopropanol

Predicting methanol binding sites

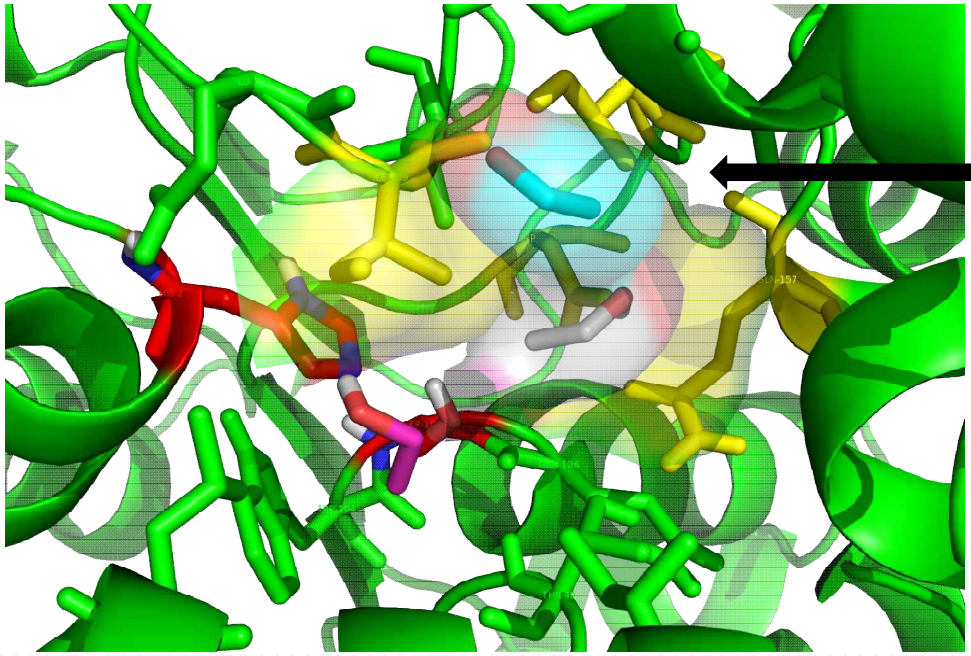
- Results
  - Binding of three ethanols in active site
  - One ethanol properly binds in small binding pocket (yellow) and two ethanols binds in large binding pocket (cyan).

➔ Ethanol binding in large binding pocket can inhibit the binding of triglyceride, which may cause the inactivation of CalB at excess methanol in production of biodiesel.



Predicting methanol binding sites

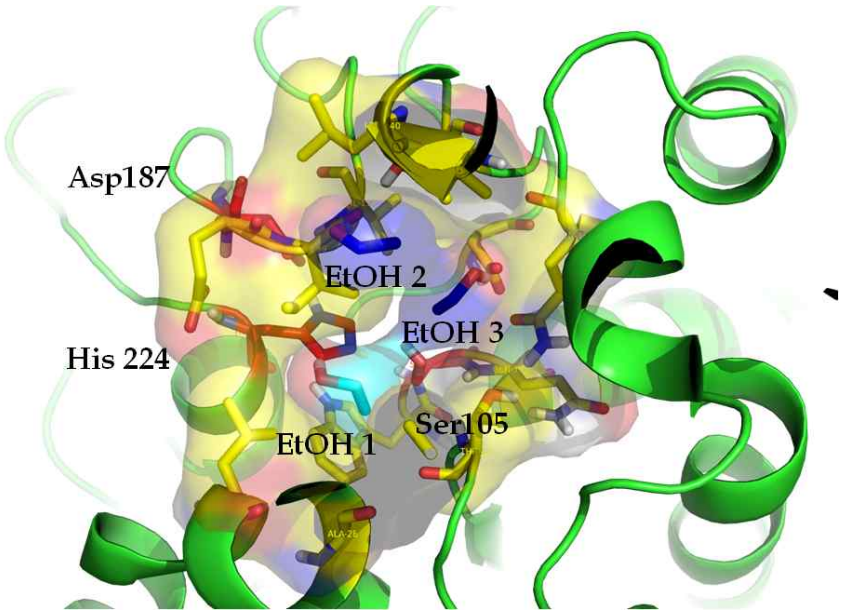
- Results
- Target residues: Interaction with two ethanols
- ➔ Hydrophobic interactions:140Leu, 141Ala, 189Ile, 190Val, 285Ile
- ➔ Hydrogen bonds: 134Glu, 138Thr, 157Gln



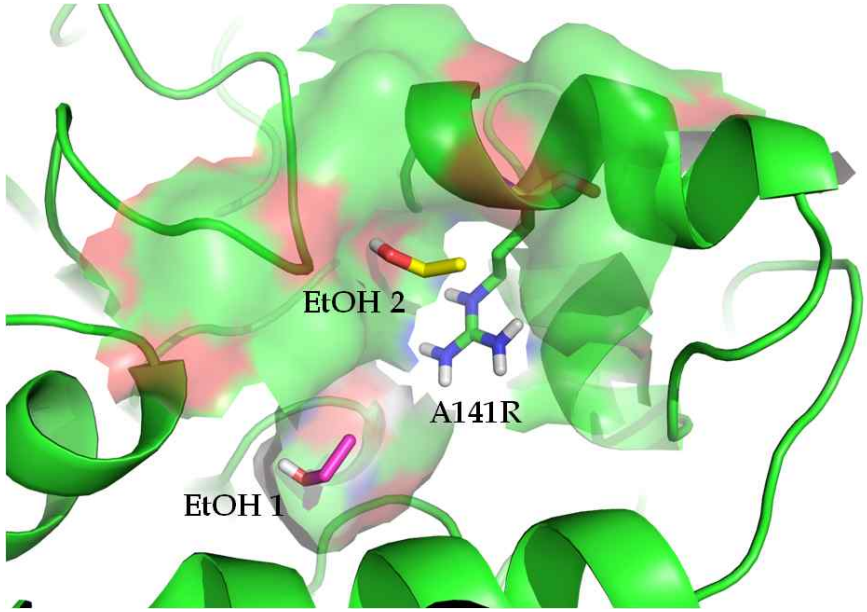
Target residues (yellow)
: contact with alcohols

Predicting methanol binding sites

- Prediction of alcohol binding
- Strategy: EtOH1(catalytic alcohol), EtOH2, EtOH2(inhibition alcohol)
- Example: A141R mutants: Removal of EtOH3 binding ➔ decreased inhibition



wild-type: 3 alcohol bindings



A141: 2 alcohols binding

Predicting methanol binding sites

		D134	T138	L140	A141	Q157	I189	V190	I285
1	Ala	x	x	x	-	x	x	x	x
2	Cys	x	x	x	x	x	x	x	x
3	Asp	-	x	x	x	x	x	x	x
4	Glu	x	x	x	x	x	x	x	x
5	Phe	x	x	x	x	x	x	x	x
6	Gly	x	x	x	x	x	x	x	x
7	His	x	x	x	O	x	O	x	x
8	Ile	x	x	x	x	O	-	x	-
9	Lys	x	O	x	O	O	x	x	x
10	Leu	x	x	-	x	x	x	x	x
11	Met	x	x	x	x	x	x	x	x
12	Asn	x	x	x	x	x	x	x	x
13	Pro	x	x	x	x	x	x	x	x
14	Gln	x	x	x	x	-	x	x	x
15	Arg	O	x	x	O	O	x	x	x
16	Ser	x	x	x	x	x	x	x	x
17	Thr	x	-	x	x	x	x	x	x
18	Val	x	x	x	x	x	x	-	x
19	Trp	x	x	x	O	x	O	x	x
20	Tyr	x	x	x	x	x	O	x	x

O : inhibition을 줄이도록 예측된 **mutants.**

Target mutants: D134R, T138K, A141H, A141K, A141R, A141W, Q157I, Q157K, Q157R, I189H, I189W, I189Y

현재 **in vitro mutation experiment** 중

Flexibility control

방법 **3. Spring model (**서울대 제안 이론**)**을 통한 **CalB**의 **flexbility** 증대 연구

- Enhancement of CalB activity by modification of enzyme flexibility
- Mutations to hydrophilic residues to induce active enzyme motion in solvents

- Methods
  - Catalytic motion prediction of CalB by spring model

Flexibility control - Spring model

- $F = kx$
  - $k \propto$ (rigidity =1/flexibility)

  = 1/ (B-factor)
  - x = (deformation distance)

  = (RMSD)

  = (C_α atom of distance between apo form and substrate-bound form)

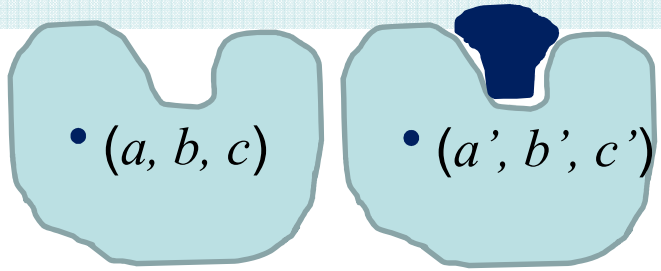
  = (a'- a, b'- b, c'- c)

- This new model uses only data from x-ray crystallography and is simple to calculate flexibility.
- Using this model, each residual flexibility is expressed by residual force relatively.

Flexibility control - Spring model

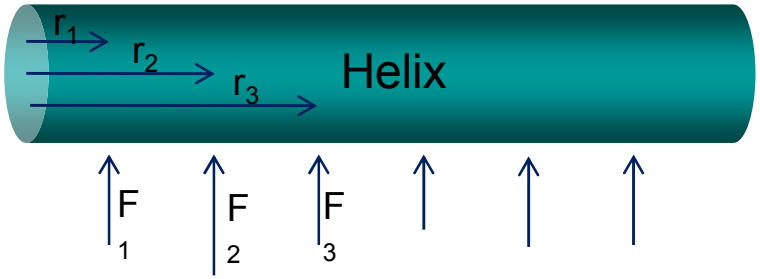
$$F = k\,x = \frac{\overrightarrow{RMSD}}{\text{B-factor}}$$

$$= \frac{(a'- a,\ b'- b,\ c'- c\ )}{\text{B-factor}}$$



- The pdb of free form and substrate bound form is superimposed using program DaliLite and RMSD is calculated.

- Torque calculation at helix edges
  - The degree of distortion was obtained by summation of each residual force cross distance from helix edge to each residue



$$\overrightarrow{\text{torque}} = \sum \vec{r} \times \vec{F} = \sum \vec{r} \times$$

r : distance from edge residue to each residue
F : residual force from spring model

Flexibility control – Spring model

- RMSD was calculated using superimposition of 1TCA (pdb file of free form) and 1LBS (pdb file of substrate bound form).
- A287 has extraordinary high torque value. The circular permutation near A287 increased enzyme activity dramatically. In this case, activity of CalB was decreased.
  Qian, Z. & Lutz, S. (2007) *ChemBioChem*

Res. #	Torque value	Res. #	Torque value	Res. #	Torque value
13	0.3383899	106	1.7379546	207	0.512569
18	0.501267	117	0.564379	211	0.774318
20	0.2194913	119	0.2524794	212	0.3909227
22	0.090343	121	0.184276	216	0.34374
33	0.2779379	125	1.9418898	226	2.2019204
37	0.696462	131	1.32531	242	0.589852
44	1.2343368	139	0.5796522	250	0.1556807
57	0.19969	141	0.31437	252	0.166899
62	0.8462247	142	0.5903451	255	0.170104
66	0.462245	146	1.218033	257	0.134847
68	0.1538915	152	0.2610305	268	1.5984291
70	0.107256	156	0.654391	287	12.11521
76	3.9162477	162	1.1012568	302	0.2099593
93	1.807374	169	0.32644	304	0.176673
99	0.5582829	179	1.411592		
104	1.405437	183	0.279093		

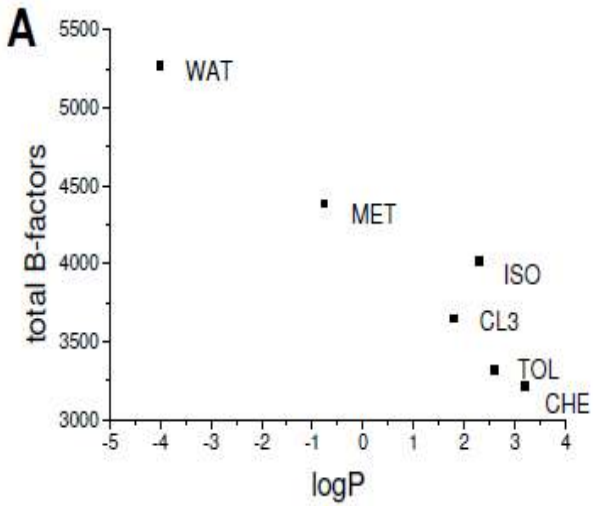
- Spring model found the hot spot of *Candida antarctica* lipase B.
- A287 is not suitable site of mutation for the activity increase w/o stability loss.
- G93, V125, T76 were considered as important sites for the motion of catalysis. The helix edges far from these sites were selected as target sites.

Flexibility control – Hydrophilic residues

- Objective
  - The MD simulation of CalB showed the hydrophilic surface was decreased in organic solvents.
  - As logP increased, total B-factor of CalB decreased. Trodler, P. & Pleiss, J. (2008) *BMC Structural Biology*

Table 1: Total and hydrophilic surface of CALB in the crystal structure and averaged over the last 1 ns of each simulation in six solvents. Hydrophilic residues are by negative Eisenberg

solvent	total surface [Å²]	hydrophilic surface [Å²]
crystal structure	12043	6071
water	12659	6410
methanol	12564	6069
chloroform	12281	5828
isopentane	12157	5858
toluene	12115	5838
cyclohexane	12014	5710



• The introduction of hydrophilic residues is needed for activity increase in organic solvents.
•Target sites from spring model were changed to hydrophilic residues. The change of volume of amino acid were minimized.

Flexibility control – Mutation

- Result
  - V139E, C216D and I255E showed increased activity.

	μg/ml	Unit/ml	specific activity (unit/mg)	% increase of activity	
Skp_CalB	44.994	0.471	10.469	100	Wild type
V139E	30.301	0.364	12.011	115	
C216D	4.774	0.058	12.240	117	
I255E	26.223	0.469	17.888	171	

❖ 활성이 좋은 **3**개의 변이주 **V139E,C216D,I255E** 를 얻음.

❖ 실제 **FAME**에 대한 실험 과 유기용매 안정성에 관한 실험 필요.