LOSCHMIDT LABORATORIES

PROTEIN ENGINEERING

7. Rational and semi-rational design

Loschmidt Laboratories
Department of Experimental Biology
Masaryk University, Brno

Outline

- Protein engineering approaches
- Semi-rational design
  - identification of hot-spots
  - evaluation of hot-spots
  - selection of substitutions
  - design of library
  - mutagenesis and screening
- Rational design
  - molecular modeling

Outline

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Protein engineering

- altering protein structure to improve its properties
- three main approaches
  - rational design
  - directed evolution
  - semi-rational design

Protein engineering approaches

RATIONAL DESIGN

1. Computer aided design

2. Site-directed mutagenesis

Individual mutated gene

3. Transformation

4. Protein expression

5. Protein purification

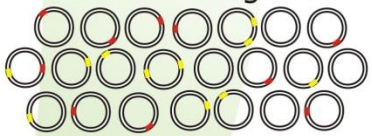
6. *not applied*

Constructed mutant enzyme

DIRECTED EVOLUTION

1. *not applied*

2. Random mutagenesis



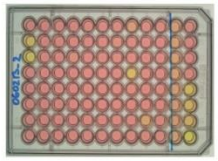
Library of mutated genes
(>10,000 clones)

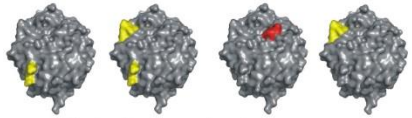
3. Transformation

4. Protein expression

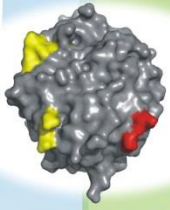
5. *not applied*

6. Screening and selection
- stability
- selectivity
- affinity
- activity





Selected mutant enzymes



IMPROVED ENZYME

7. Biochemical testing

Protein engineering approaches

	Rational design	Directed evolution	Semi-rational design
high-throughput screening/selection	not essential	essential	advantageous but not essential
structural and/or functional information	both essential	neither essential	either is sufficient
sequence space exploration	low	high, random	moderate, targeted
probability to obtain synergistic mutations	moderate	low	high

Structural information

- **worldwide Protein Data Bank (wwPDB)**
  - http://www.wwpdb.org/
  - central repository of ~160,000 experimental macromolecular structures
- **RCSB PDB**
  - https://www.rcsb.org/
- **PDBe**
  - https://www.ebi.ac.uk/pdbe/
- **PDBj**
  - https://pdbj.org/

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  - mutagenesis and screening
- Rational design
  - molecular modeling

Semi-rational design

- combine advantages of rational and random approaches
- selection of promising target sites (hot-spots) → mutagenesis → creation of small "smart" libraries
- based on knowledge of protein structure and function
- ☺ high-throughput screening usually not needed
- ☺ increased chance of obtaining variants with desired properties
- ☹ certain knowledge of protein structure-function relationships is still required, ☺ but not that much

- Protein engineering approaches
- Semi-rational design
  - identification of hot-spots
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Identification of hot-spots

- hot-spots for engineering catalytic properties
- hot-spots for engineering thermostability

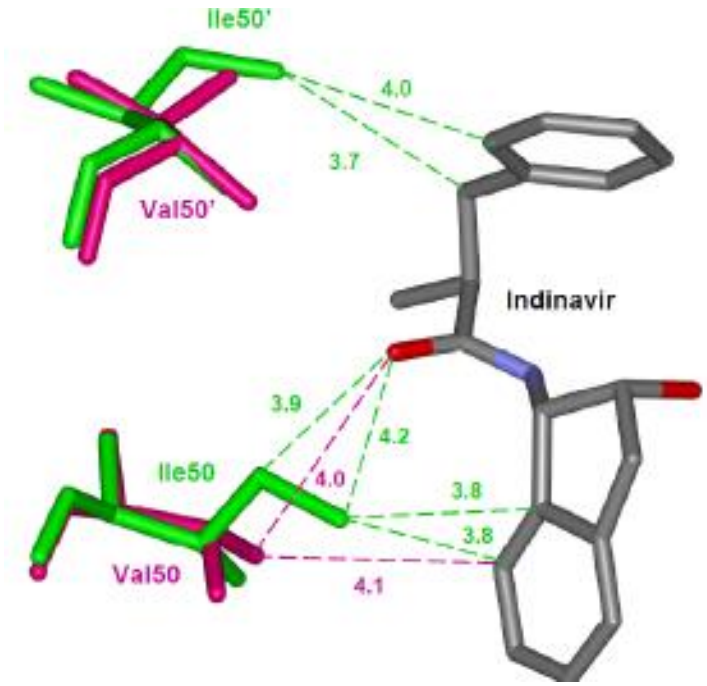
Hot-spots for engineering catalytic properties

- residues mediating substrate binding, transition-state stabilization or product release → mutations can improve or disrupt binding, catalysis or ligand transport
  - residues involved in protein-ligand interactions
  - residues located in binding pockets
  - residues located in access tunnels

→ these residues also include catalytic or other essential residues which generally should not be mutated!

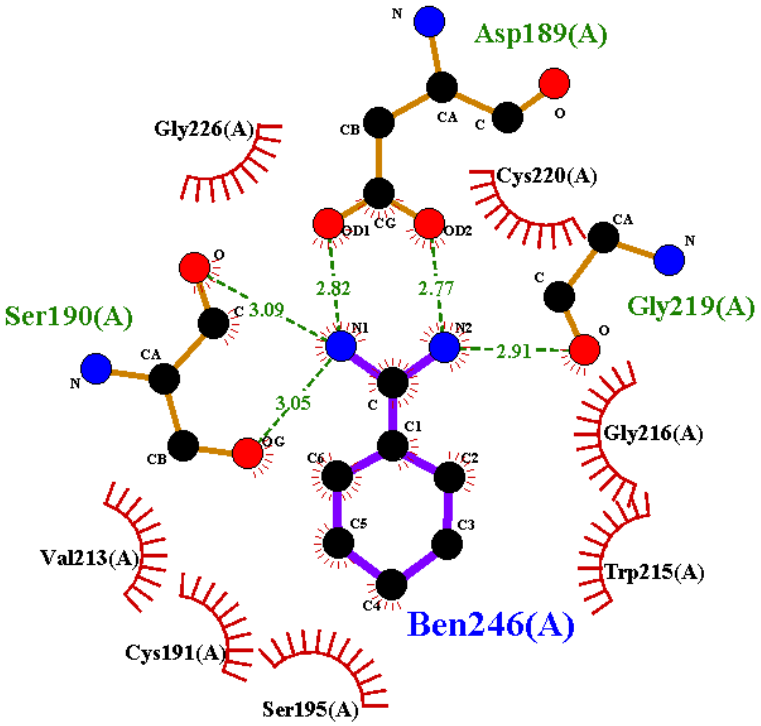
Analysis of protein-ligand interactions

- requires 3D structure of protein-ligand complex
  - experimental structure (wwPDB)
  - theoretical model (molecular docking)

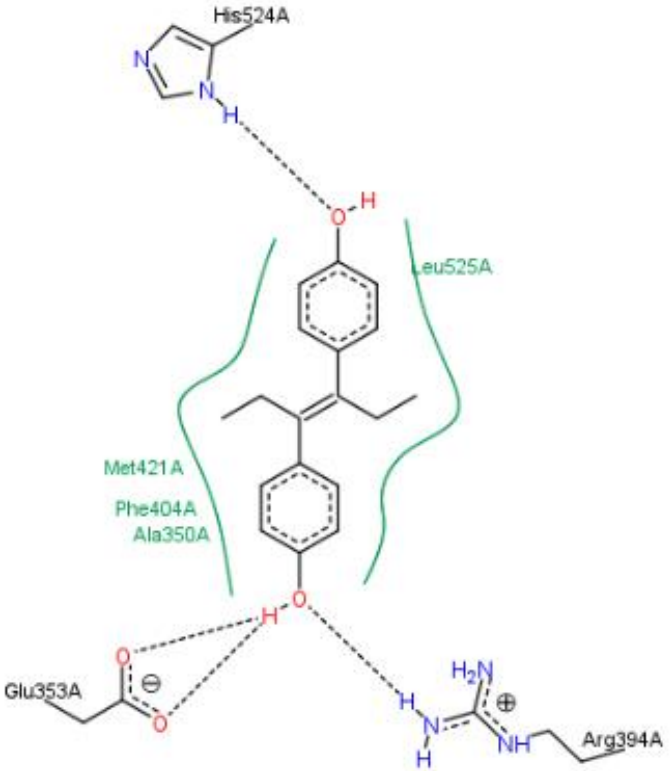


Analysis of protein-ligand interactions

- schematic diagrams of protein-ligand interactions



LigPlot, LigPlot+

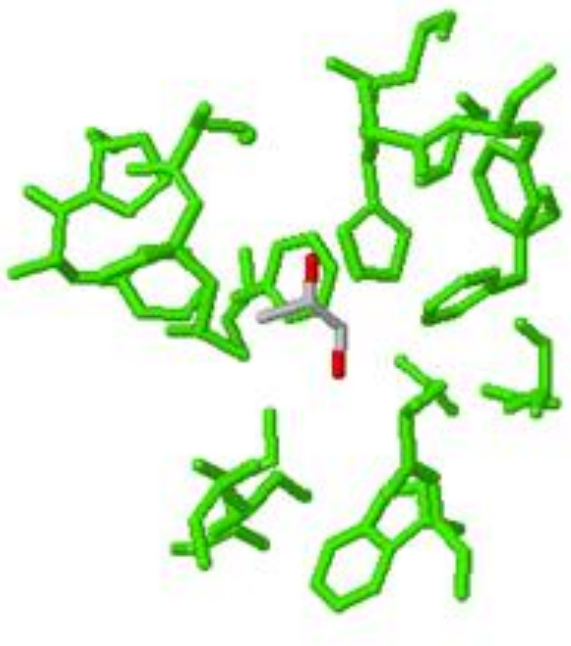


PoseView

Analysis of protein-ligand interactions

- inter-atomic contacts between protein and bound ligands

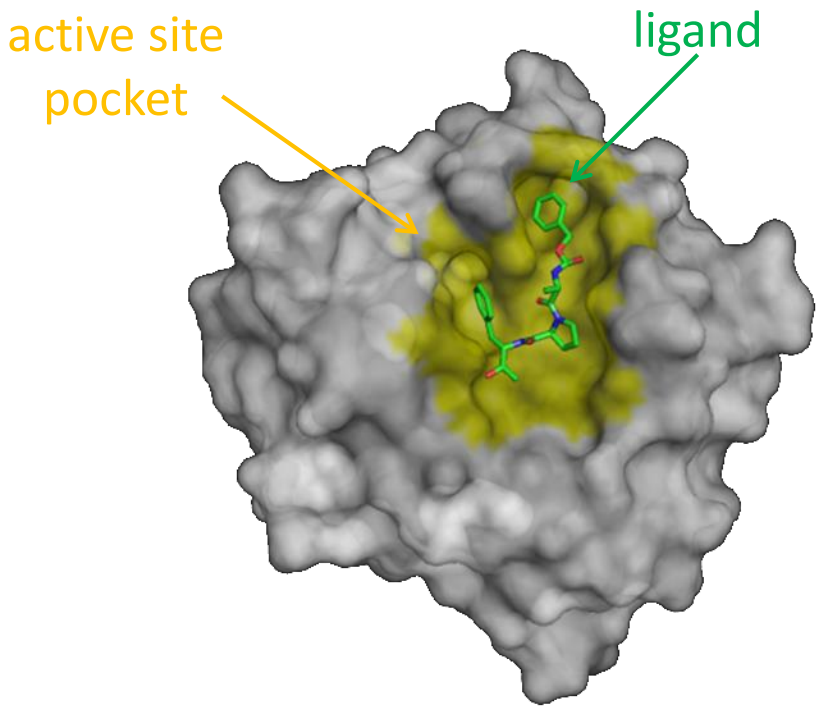
Residue		Dist(Å)	Surf(Å^2)	Number of contacts
ASN	38 A	2.7	22.5	2
ASP	108 A	2.8	35.1	5
ILE	134 A	6.3	0.7	1
PHE	143 A	5.0	6.5	2
PHE	151 A	3.3	26.7	4
PHE	169 A	3.5	6.4	2
VAL	173 A	3.6	23.4	1
LEU	177 A	4.8	8.5	2
ILE	211 A	5.2	3.8	1
LEU	248 A	5.6	10.3	4
HIS	272 A	3.8	33.8	9
PHE	273 A	3.5	2.3	2
BR	901 A	3.8	30.7	2



LPC server

Analysis of binding pockets

- binding and active sites of enzymes are often associated with structural pockets and cavities



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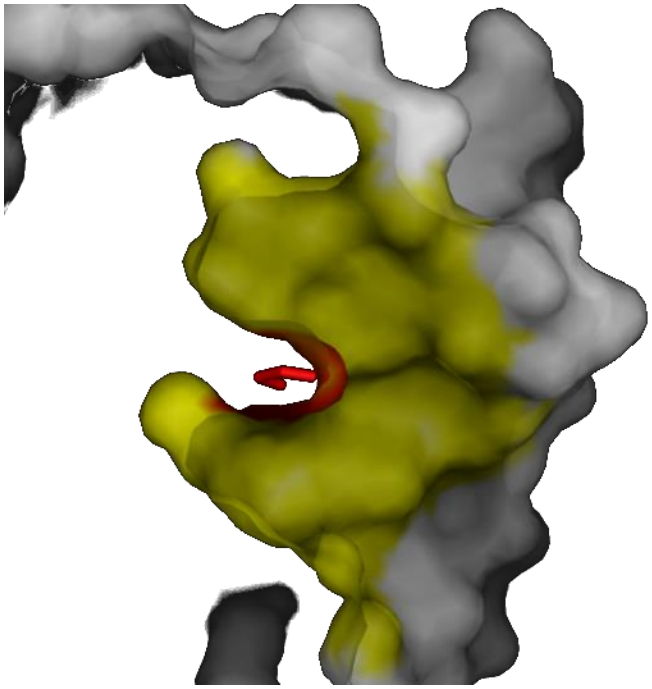

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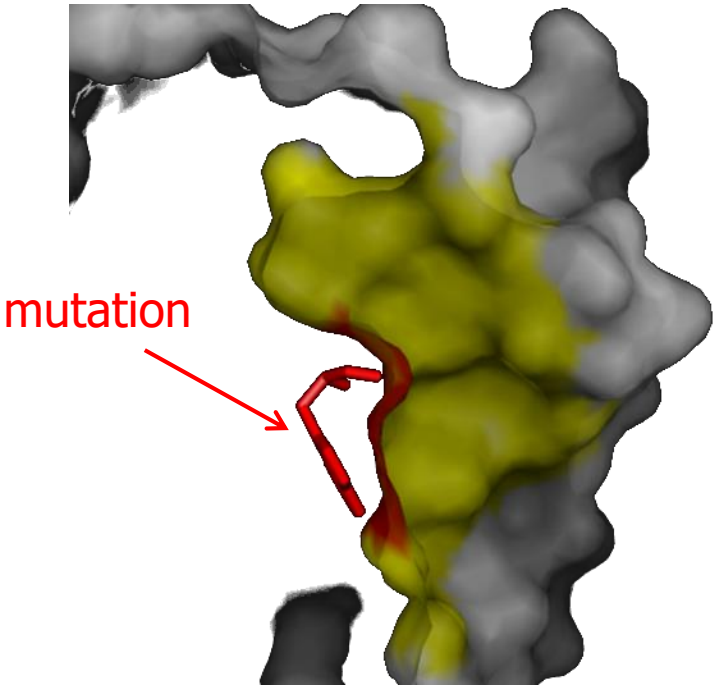

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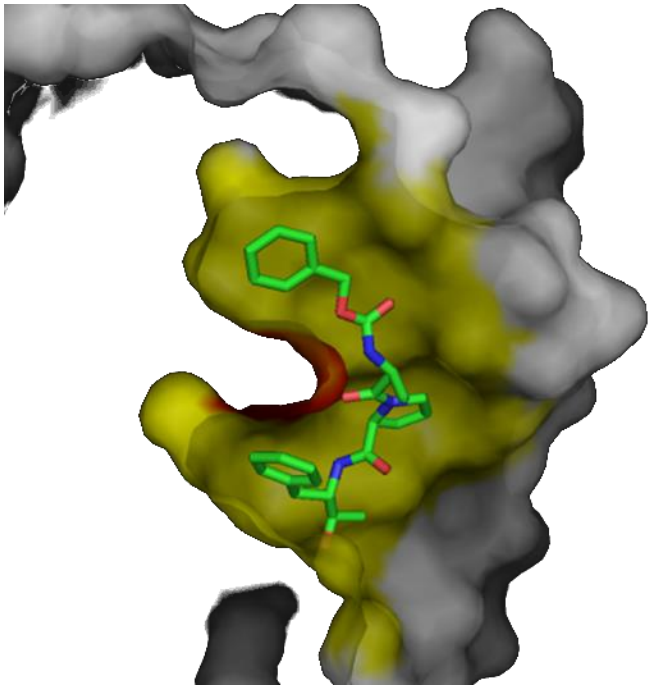

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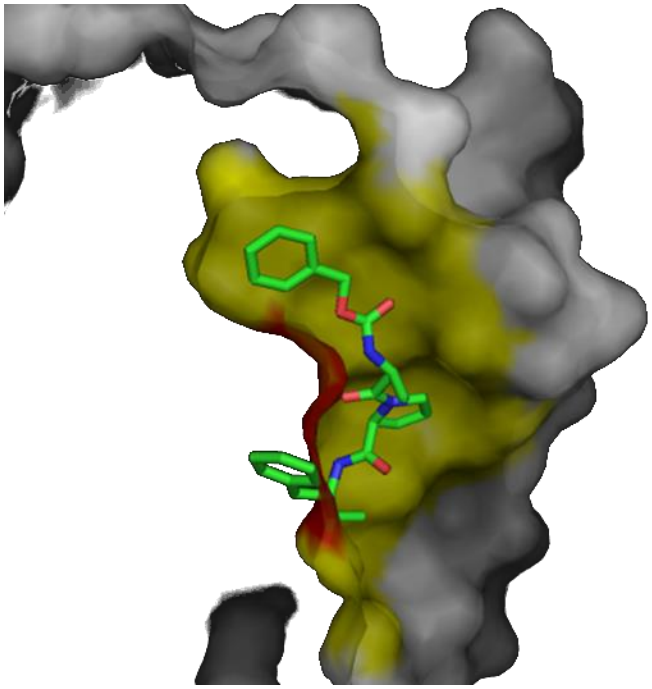
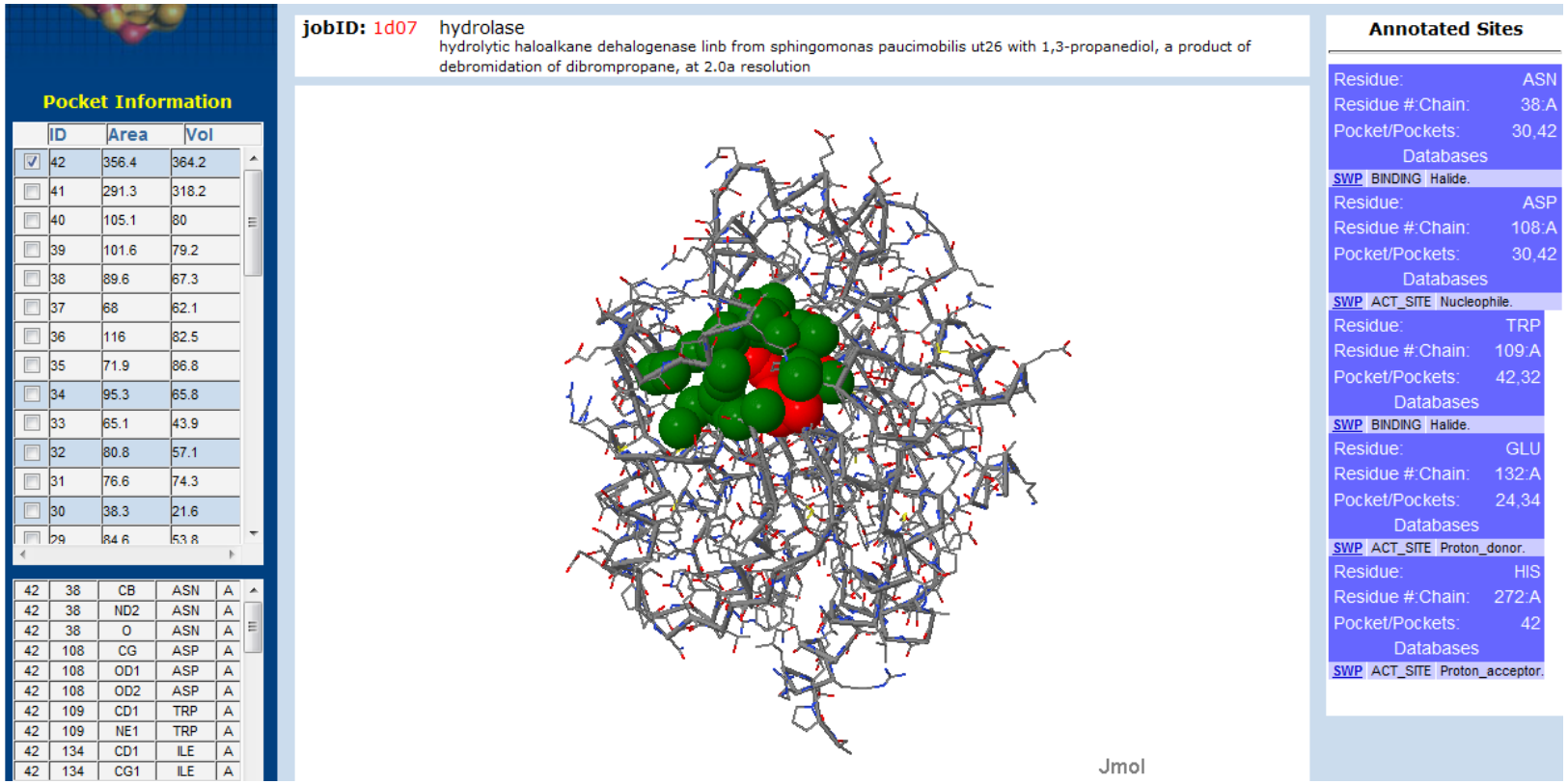


Analysis of binding pockets

- binding and active sites of enzymes are often associated with structural pockets and cavities
  - most amino acid residues located in these pockets may come into contact with the ligands during the catalytic cycle
  - → one can accurately predict which residues may interact with the ligand even without precise knowledge of ligand orientation in the active site
- requires 3D structure of protein
- software for detection of pockets
  - CASTp, fPocket, MetaPocket, Caver Analyst…

Analysis of binding pockets

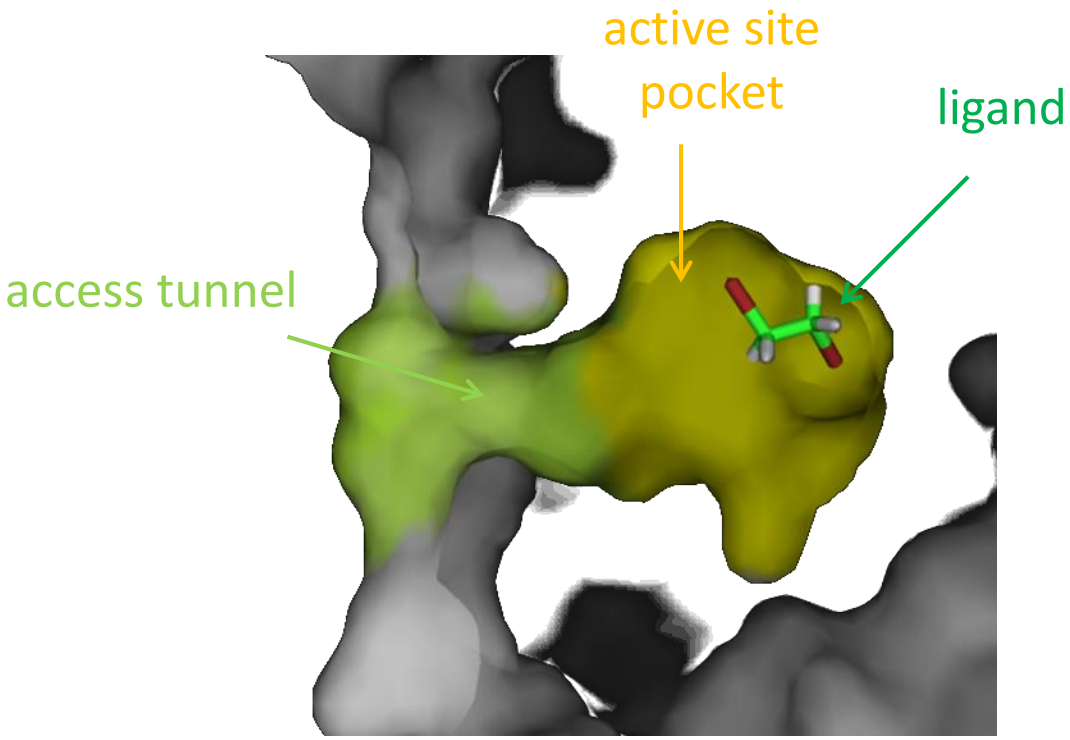
- detailed characterization of all pockets in the structure



CASTp

Analysis of access tunnels

- buried binding or active sites are connected with bulk solvent by access tunnels



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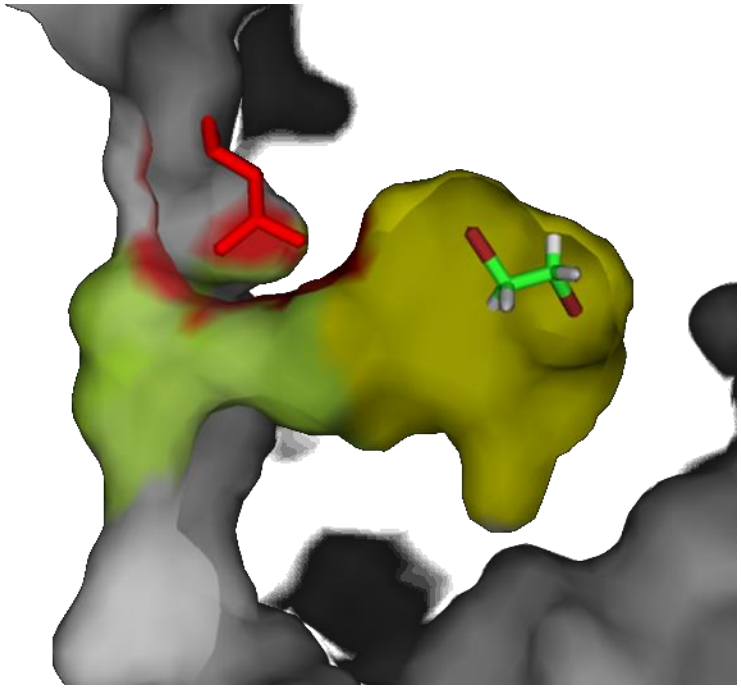

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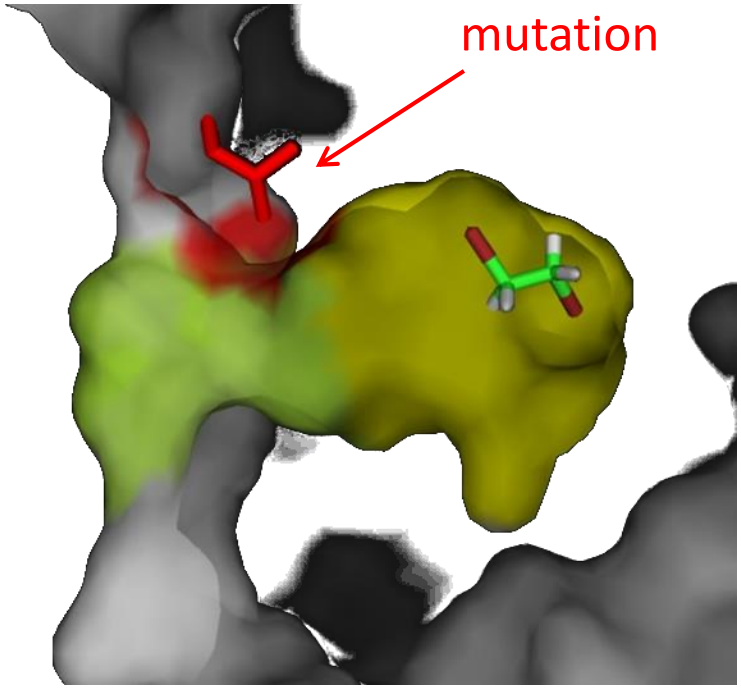

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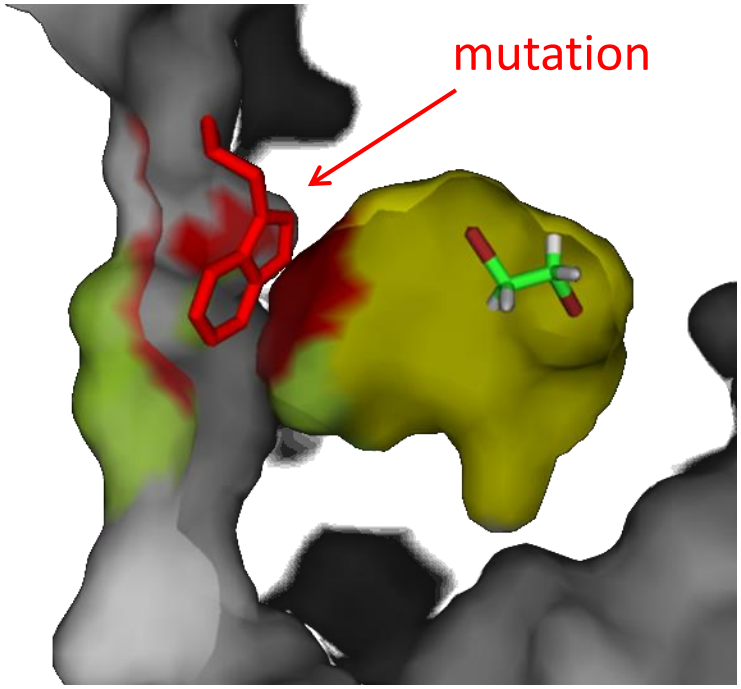
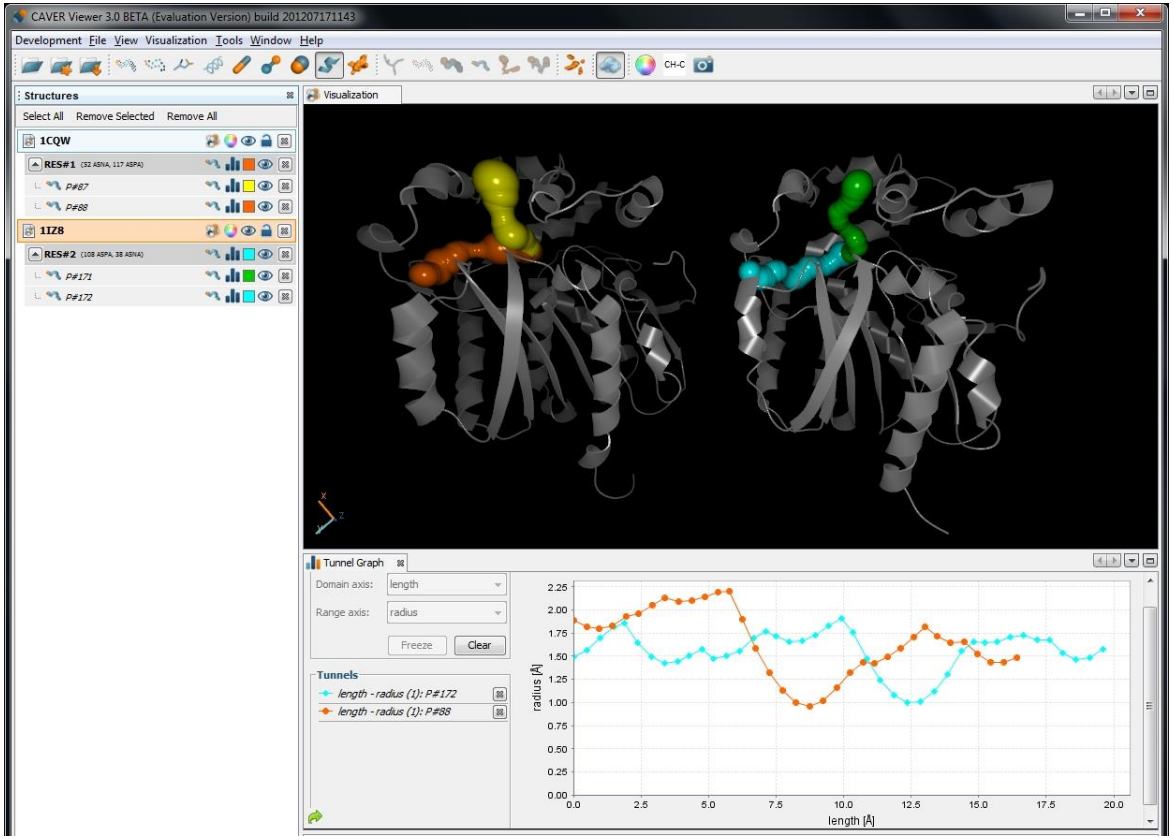


Analysis of access tunnels

- buried binding or active sites are connected with bulk solvent by access tunnels
  - adjusted to permit transport of specific molecules
  - mutations can speed-up or hinder transport of molecules as well as allow transport of other molecules
- requires 3D structure of protein
- software for detection of tunnels
  - Caver, Mole, HOLE, PoreWalker

Analysis of access tunnels

- detailed characteristics of access tunnels



CAVER Analyst 2.0

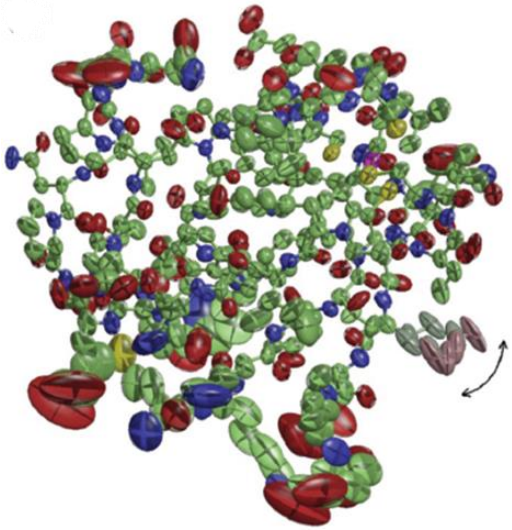
Hot-spots for engineering thermostability

- highly flexible residues – introduction of rigidifying mutations
- residues located in access tunnels
- residues predicted by systematic *in silico* saturation mutagenesis

→ these residues may also include catalytic or other essential residues which generally should not be mutated!

Identification of highly flexible residues

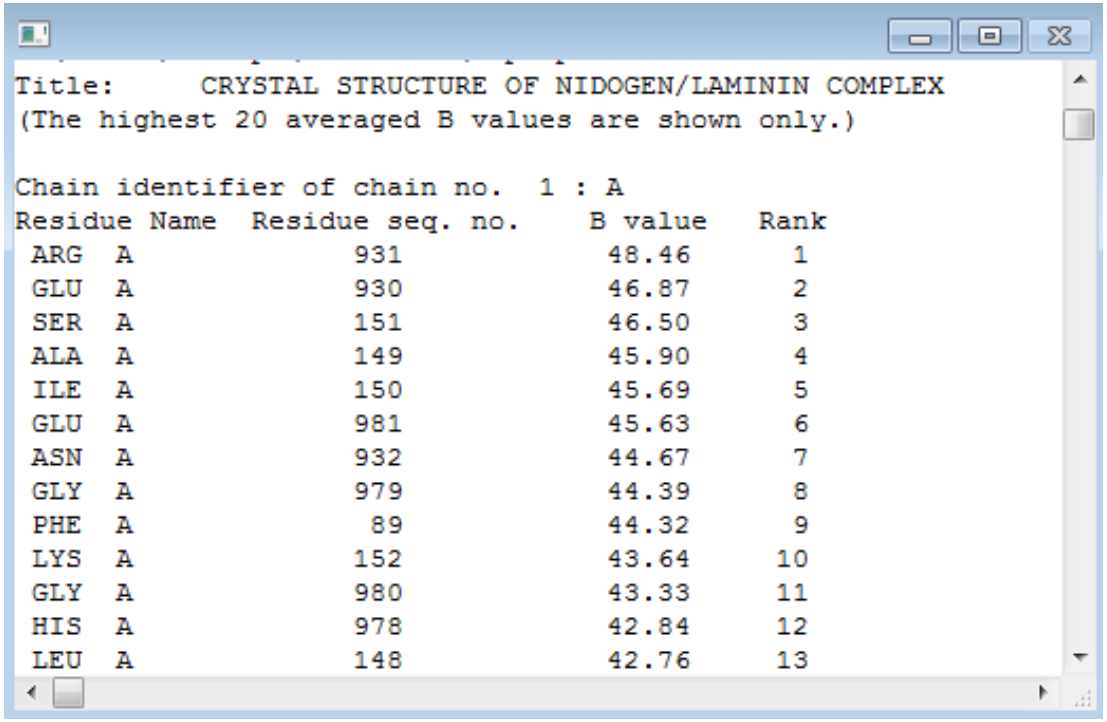
- prediction based on crystallographic B-factors
  - reflect the degree of thermal motion, and thus the flexibility of individual residues



- requires 3D structure of protein
  - experimental structure determined by X-ray crystallography (wwPDB)

Identification of highly flexible residues

- average B-factor of each residue in the target protein



Title: CRYSTAL STRUCTURE OF NIDOGEN/LAMININ COMPLEX
(The highest 20 averaged B values are shown only.)

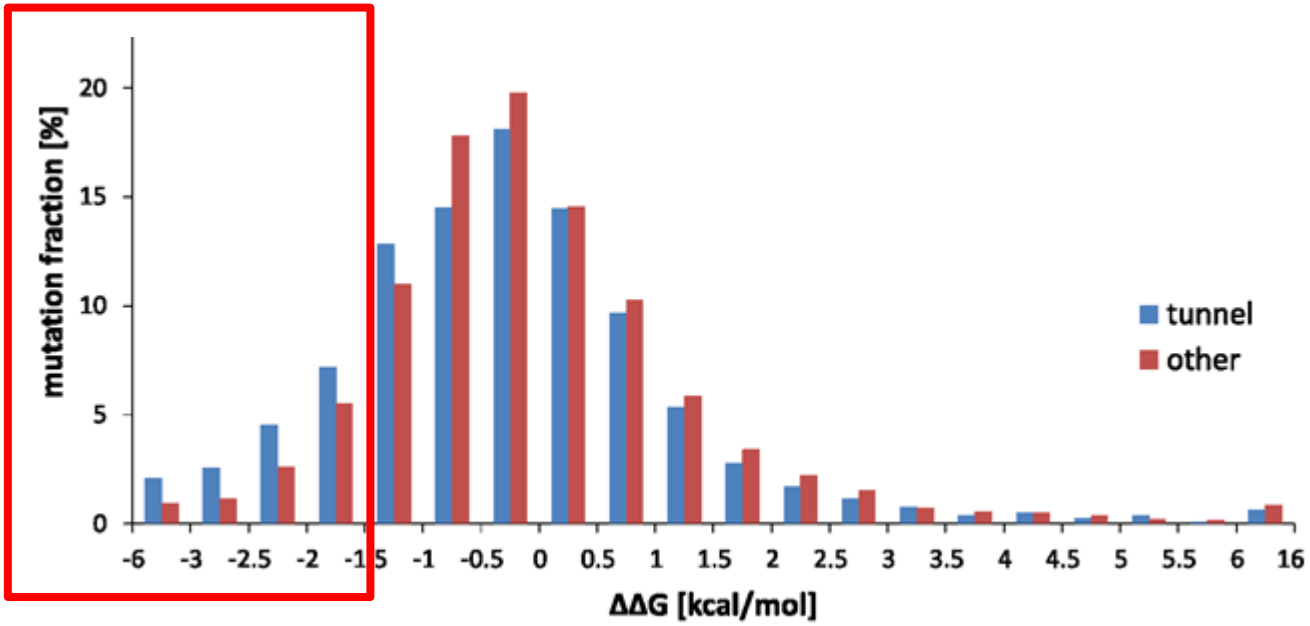
Chain identifier of chain no. 1 : A

Residue Name		Residue seq. no.	B value	Rank
ARG	A	931	48.46	1
GLU	A	930	46.87	2
SER	A	151	46.50	3
ALA	A	149	45.90	4
ILE	A	150	45.69	5
GLU	A	981	45.63	6
ASN	A	932	44.67	7
GLY	A	979	44.39	8
PHE	A	89	44.32	9
LYS	A	152	43.64	10
GLY	A	980	43.33	11
HIS	A	978	42.84	12
LEU	A	148	42.76	13

B-FITTER

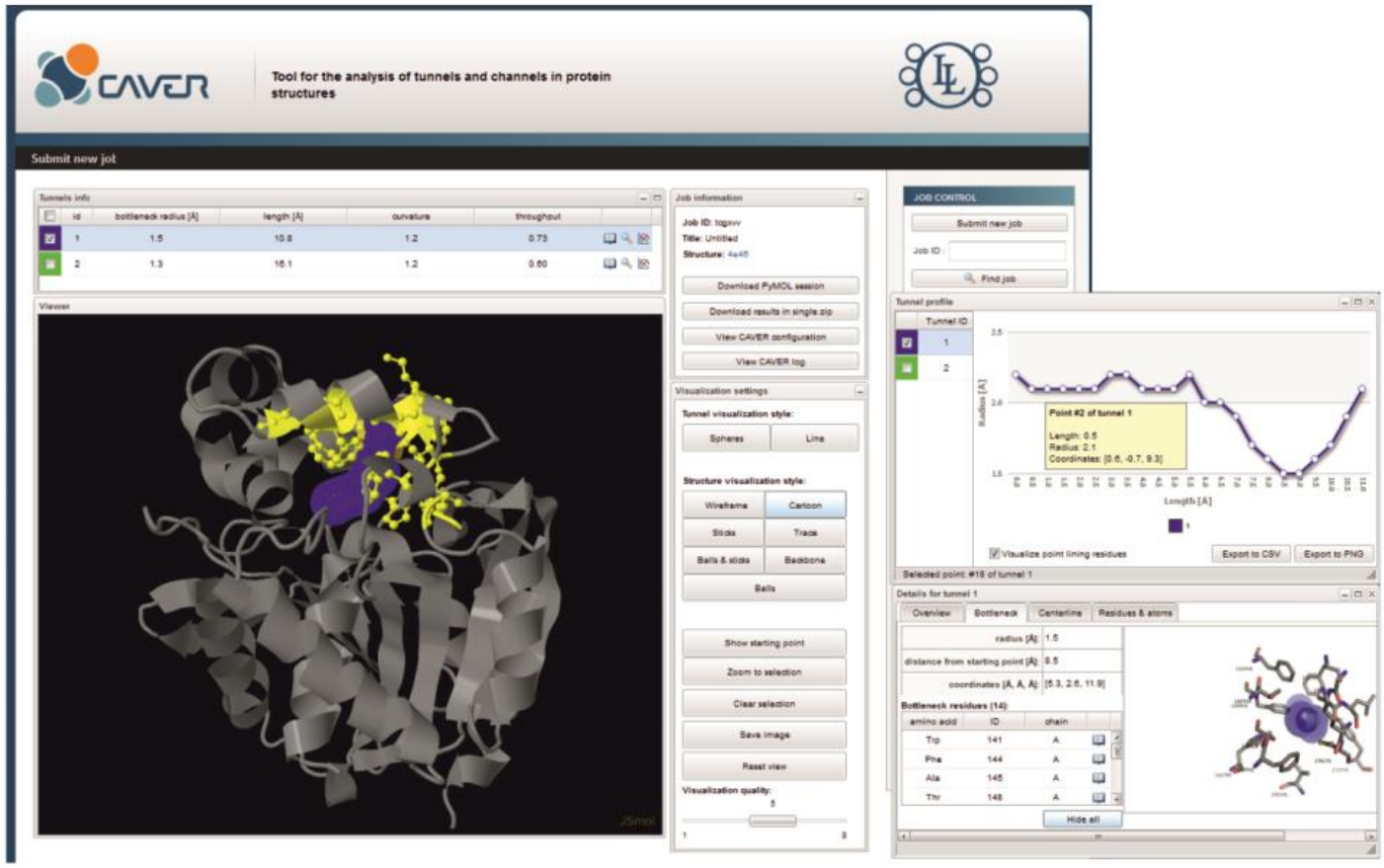
Analysis of access tunnels

- saturation mutagenesis in tunnel residues has 2× better chance to significantly improve stability than mutagenesis in other protein regions (based on computational predictions)



Analysis of access tunnels

- Detection of tunnels in proteins and analysis of ligand transport



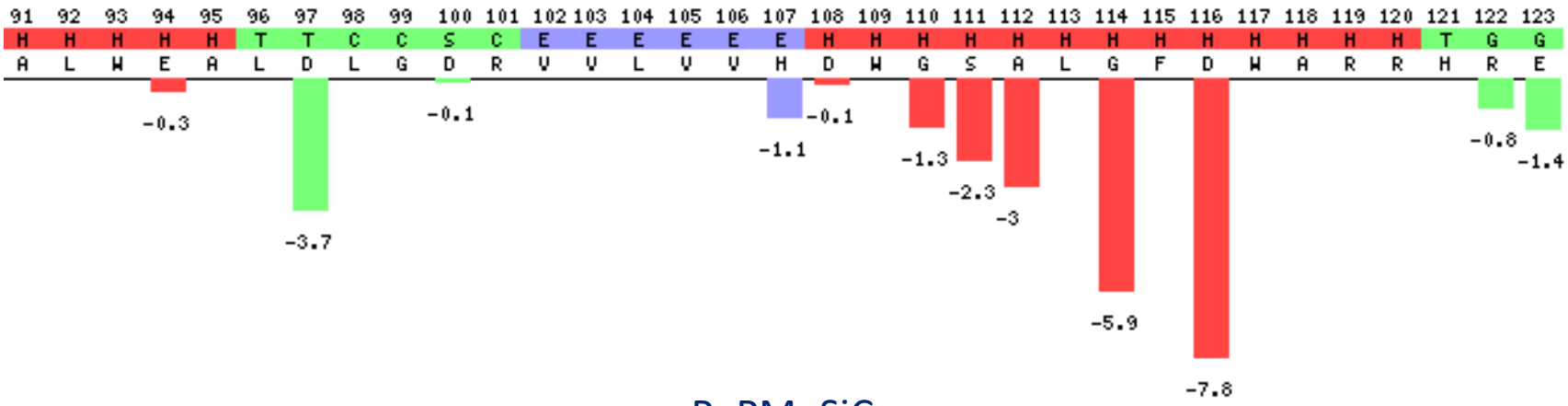
CAVER Web

Systematic *in silico* saturation mutagenesis

- computational tools for the prediction of effect of amino acid substitutions on protein stability
  - each residue in the protein structure is replaced by all other possible amino acids and the change in folding free energy (ΔΔG) upon mutation is estimated
  - positions with a high proportion of stabilizing mutations and/or low proportion of destabilizing mutations are good candidates for randomization by experimental saturation mutagenesis
- usually requires 3D structure of protein
  - experimental structure (wwPDB)
  - theoretical model (homology modeling)

Systematic *in silico* saturation mutagenesis

- fast systematic scan of all possible single-point mutations – prediction of stability changes upon mutation
- sequence optimality score (the sum of all negative ΔΔGs at a given position) – indicates poorly optimized positions



PoPMuSiC

- Protein engineering approaches
- Semi-rational design
  - identification of hot-spots
  - evaluation of hot-spots
  - selection of substitutions
  - design of library
  - mutagenesis and screening
- Rational design
  - molecular modeling

Evaluation of hot-spots

- hot-spots identified by computational tools can be further evaluated to prevent replacing indispensable amino acid residues and to prioritize the hot-spots (i.e., order the hot-spots based on their suitability for mutagenesis)

- analysis of evolutionary conservation
- prediction of effects of mutations on protein stability or function

Analysis of evolutionary conservation

- residues essential for maintaining structural or functional properties of a protein tend to be conserved during evolution
  - conserved residues are generally not recommended as suitable targets for mutagenesis - their replacement often leads to the loss of protein function
  - mutagenesis targeting highly mutable positions provides a significantly higher proportion of viable variants than random mutagenesis
  - targeting moderately or highly variable positions, which are expected to be tolerant to a wide range of substitutions, represents a good approach for producing efficient smart libraries (i.e., libraries with a high proportion of correctly folded and active variants)

Analysis of evolutionary conservation

- residue conservation can be derived from a multiple alignment of a set of related proteins (3D structure not required)

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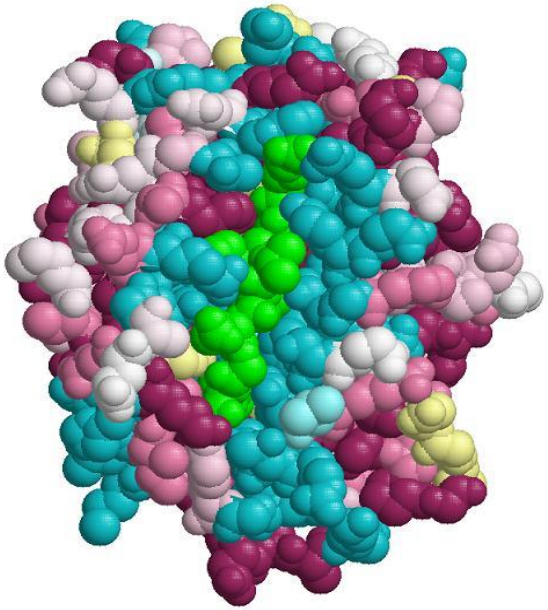
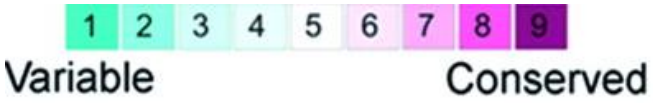
Analysis of evolutionary conservation

- evolutionary conservation of individual positions in protein mapped on protein 3D structure





ConSurf

Prediction of mutation effects

- computational tools for the prediction of effect of amino acid substitutions on protein stability or protein function
  - *in silico* site-saturation mutagenesis of identified hot-spots – check if mutations at a given site are likely to be tolerated
  - many highly destabilizing/deleterious mutations predicted for a certain position – given site is not a very good target for mutagenesis

  - sites with only a few highly destabilizing /deleterious mutations predicted can still represent promising hot-spots (the amino acids with potentially destabilizing/deleterious effects can be discarded from the library by the appropriate selection of degenerate codons)

Prediction of mutation effects

- effects on protein stability – usually requires 3D structure of protein
  - experimental structure (wwPDB)
  - theoretical model (homology modeling)

- effects on protein function – sequence information often sufficient

Prediction of mutation effects

- prediction of effect of substitutions on protein stability
  - Evaluation of the change of protein free energy upon mutation
  - Evaluation of contributions of individual interactions to total energy
  - Usually requires structural information
- software for prediction of effect of mutation on stability
  - Rosetta, FoldX, CUPSAT, ERIS

Prediction of mutation effects

- prediction of effect of substitutions on protein stability

Amino Acid Mutations			
Amino acid	Overall Stability	Torsion	Predicted ΔΔG (kcal/mol)
GLY	Stabilising	Unfavourable	1.48
ALA	Destabilising	Unfavourable	-0.9
VAL	Destabilising	Unfavourable	-2.23
ILE	Destabilising	Unfavourable	-2.12
MET	Stabilising	Unfavourable	1.89
PRO	Stabilising	Unfavourable	1.55
TRP	Stabilising	Favourable	2.73
SER	Stabilising	Unfavourable	1.2
THR	Destabilising	Unfavourable	-0.44
PHE	Stabilising	Favourable	3.64
GLN	Destabilising	Unfavourable	-0.69
LYS	Stabilising	Unfavourable	9.91
TYR	Stabilising	Favourable	0.96
ASN	Stabilising	Favourable	4.14
CYS	Destabilising	Favourable	-6.73
GLU	Stabilising	Unfavourable	4.98
ASP	Stabilising	Favourable	1.31
ARG	Stabilising	Unfavourable	2.94
HIS	Stabilising	Favourable	1.38

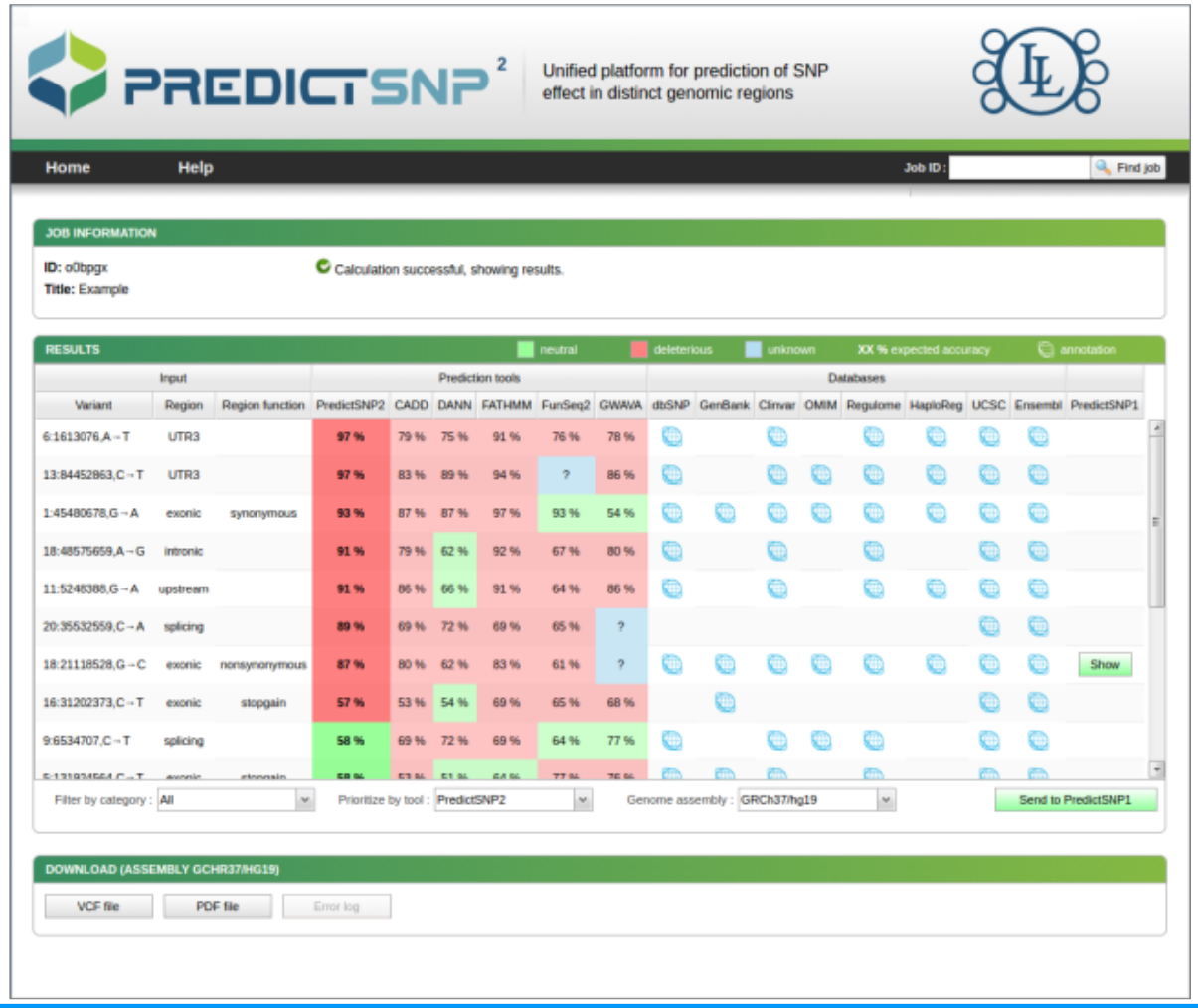
CUPSAT

Prediction of mutation effects

- prediction of effect of substitutions on protein function
  - Evaluation if a mutation would impair protein function
  - Hard to describe by physico-chemical properties > machine learning
  - Usually sequence based calculation
- software for prediction of effect of mutation on function
  - PredictSNP, SIFT, MAPP, PhD-SNP…

Prediction of mutation effects

- prediction of effect of substitutions on protein function



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Selection of substitutions

- substitutions introduced using degenerate codons
  - e.g., NNK (N = A/T/G/C; K = T/G)

IUPAC Nucleotide Nomenclature Table

symbol	base	symbol	base
A	adenosine	M	A C (amino)
C	cytidine	S	G C (strong)
G	guanine	W	A T (weak)
T	thymidine	B	G T C
U	uridine	D	G A T
R	G A (purine)	H	A C T
Y	T C (pyrimidine)	V	G C A
K	G T (keto)	N	A G C T (any)

Selection of substitutions

- all possible substitutions - NNK or NNS degenerate codons
  - ☺ encode all 20 amino acids with the lowest redundancy and price (mixture of 32 codons)
  - ☹ redundancy is not completely eliminated (3× Arg, Leu, Ser, 2× Ala, Gly, Pro, Thr and Val)

Selection of substitutions

- all possible substitutions - NNK or NNS degenerate codons
- introduction of only selected substitutions using degenerate codons encoding reduced amino acid alphabets
  - ☹ do not encode all 20 amino acids
  - ☺ decreased library size → improved screening efficiency
  - NDT – balanced set of 12 amino acids (12 codons)

Selection of substitutions

- all possible substitutions - NNK or NNS degenerate codons
- introduction of only selected substitutions using degenerate codons encoding reduced amino acid alphabets

Table 1. Oversampling necessary for 95% coverage as a function of NNK and NDT codon degeneracy.

No.[a]	NNK		NDT	
	Codons	Transformants needed	Codons	Transformants needed
1	32	94	12	34
2	1028	3066	144	430
3	32768	98163	1728	5175
4	1048576	3141251	20736	62118
5	33554432	100520093	248832	745433
6	$>1.0\times10^{9}$	$>3.2\times10^{9}$	$>2.9\times10^{8}$	$>8.9\times10^{6}$
7	$>3.4\times10^{10}$	$>1.0\times10^{11}$	$>3.5\times10^{9}$	$>1.1\times10^{8}$
8	$>1.0\times10^{12}$	$>3.3\times10^{12}$	$>4.2\times10^{10}$	$>1.3\times10^{9}$
9	$>3.5\times10^{13}$	$>1.0\times10^{14}$	$>5.1\times10^{11}$	$>1.5\times10^{10}$
10	$>1.1\times10^{15}$	$>3.4\times10^{15}$	$>6.1\times10^{12}$	$>1.9\times10^{11}$

[a] Number of aa positions at one site.

Selection of reduced amino acid alphabets

- introduction of amino acids exhibiting certain properties
  - VRK – 8 hydrophilic amino acids (12 codons)
  - NYC – 8 hydrophobic amino acids (8 codons)
  - KST – 4 small amino acids (4 codons)
  - ...

Selection of reduced amino acid alphabets

- introduction of amino acids exhibiting certain properties
- introduction of a balanced set of amino acids
  - NDT – balanced set of 12 amino acids (12 codons)

Selection of reduced amino acid alphabets

- introduction of amino acids exhibiting certain properties
- introduction of a balanced set of amino acids
- introduction of substitutions existing (at a given site) in known natural proteins
  - likely increasing the proportion of viable variants in the resulting library
  - can be obtained by analysis of multiple sequence alignment

Selection of reduced amino acid alphabets

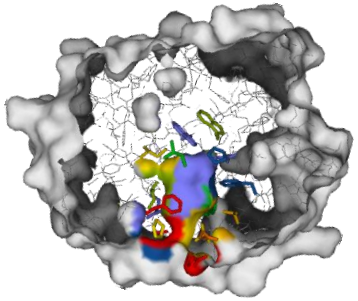
- introduction of amino acids exhibiting certain properties
- introduction of a balanced set of amino acids
- introduction of substitutions existing (at a given site) in known natural proteins
- discarding amino acids with potentially destabilizing/ deleterious effects
  - can be obtained by prediction of effects of mutations on protein stability or function

HotSpot Wizard

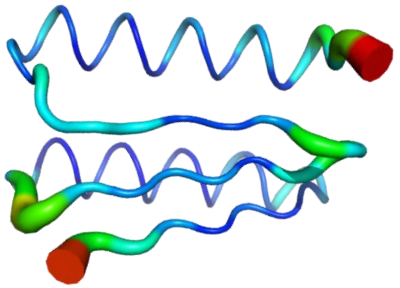
- meta-server combining several tools
  - automatic identification of hot-spots for engineering of enzyme catalytic properties
  - prioritization of hot-spots by their mutability
  - distribution of amino acids at individual positions

HotSpot Wizard

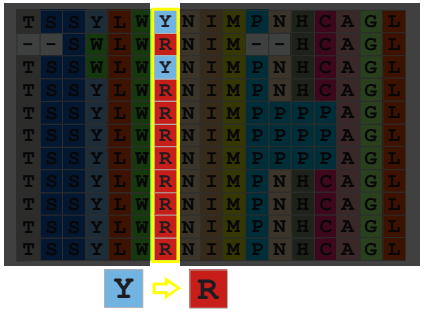
Functional hot-spots



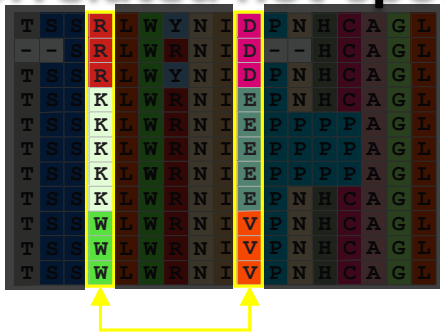
Stability hot-spots (flexibility)



Stability hot-spots (evolution)



Correlated hot-spots

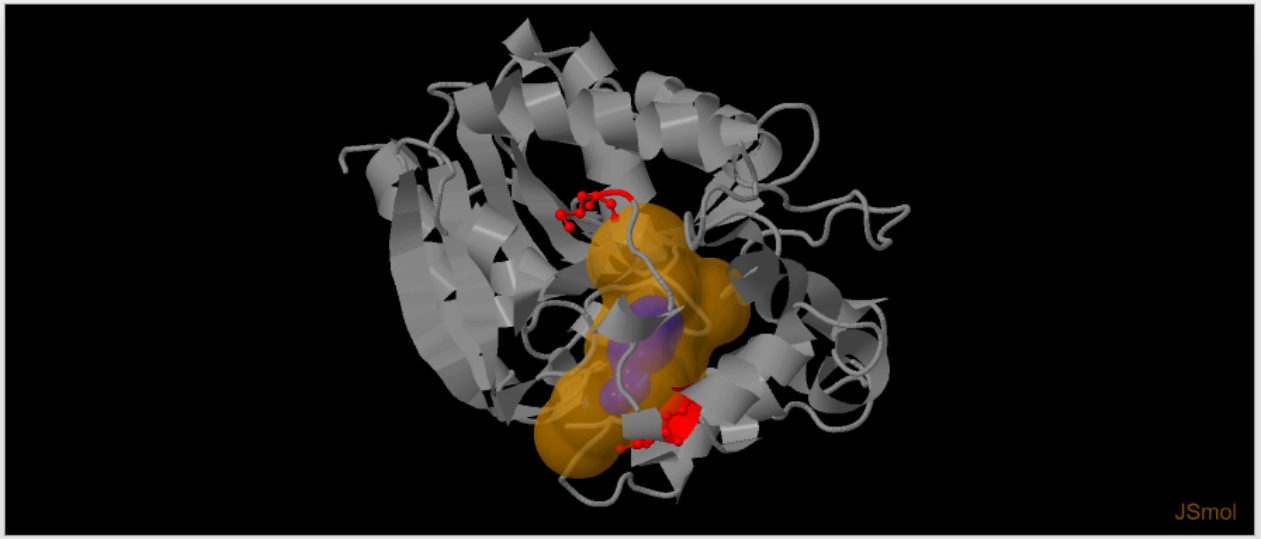


HotSpot Wizard

Functional hot spots of 1CV2

Return to Results browser

Viewer



Visualization settings

Tunnels

id	length (Å)	bottleneck radius (Å)
Starting from pocket: 1		
1	7.7	1.5

Pockets

id	chain(s)	relevance (%)	volume (Å^3)
1	A	100	576
2	A	82	883
3	A	62	275
4	A	28	753
5	A	25	183
6	A	19	119
7	A	19	632

Residue features

Exclude correlated positions | Exclude catalytic pockets | Exclude tunnels | Exclude α-helices and β-sheets | Show all residues

Exclude buried residues | Include residues with moderate mutability

chain	position	residue	mutable	non-essential	in tunnel	in catalytic pocket	HotSpot
Chain A							
A	146	Gln	✓	✓	✓	✓	✓
A	136	Met	✓	✓	✗	✓	✓
A	147	Asp	✓	✓	✓	✓	✓
A	271	Ala	✓	✓	✓	✓	✓
A	138	Ile	✓	✓	✗	✓	✓
A	247	Ala	✓	✓	✓	✓	✓
A	248	Leu	✓	✓	✓	✓	✓
A	249	Thr	✓	✓	✓	✗	✓
A	253	Met	✓	✓	✗	✓	✓

Residues selected for mutagenesis

Zoom residues | Design library

chain	position	residue	HotSpot
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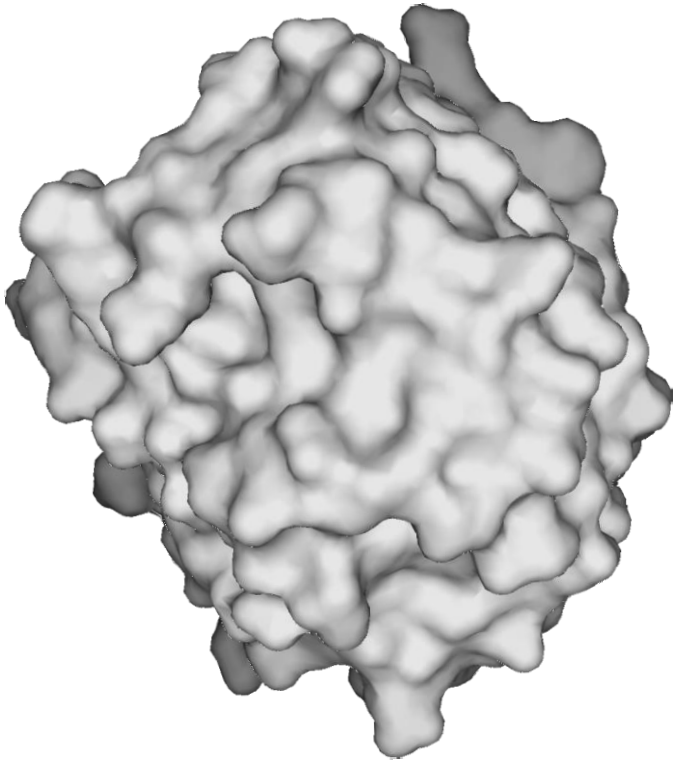
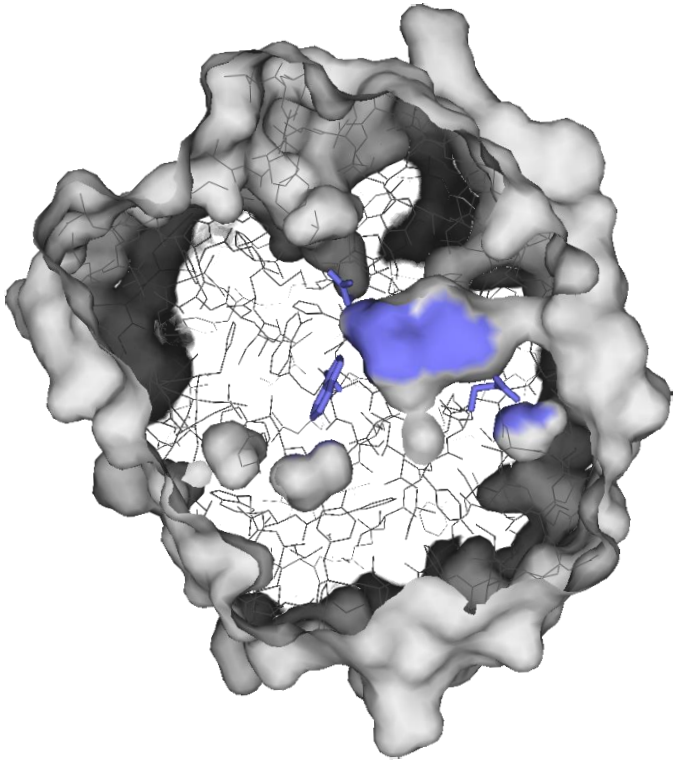
HotSpot Wizard

1. protein structure



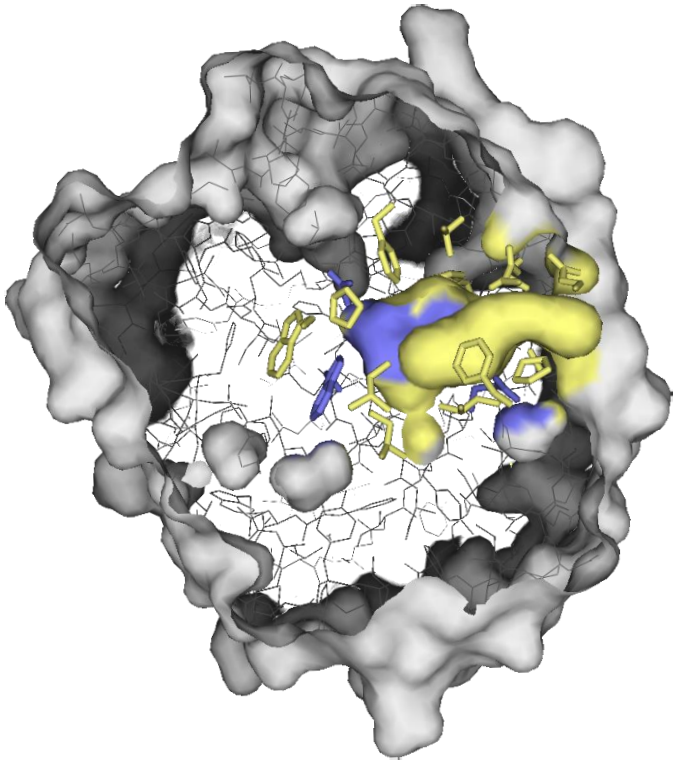
HotSpot Wizard

2. residues indispensable for protein function: catalytic and binding residues



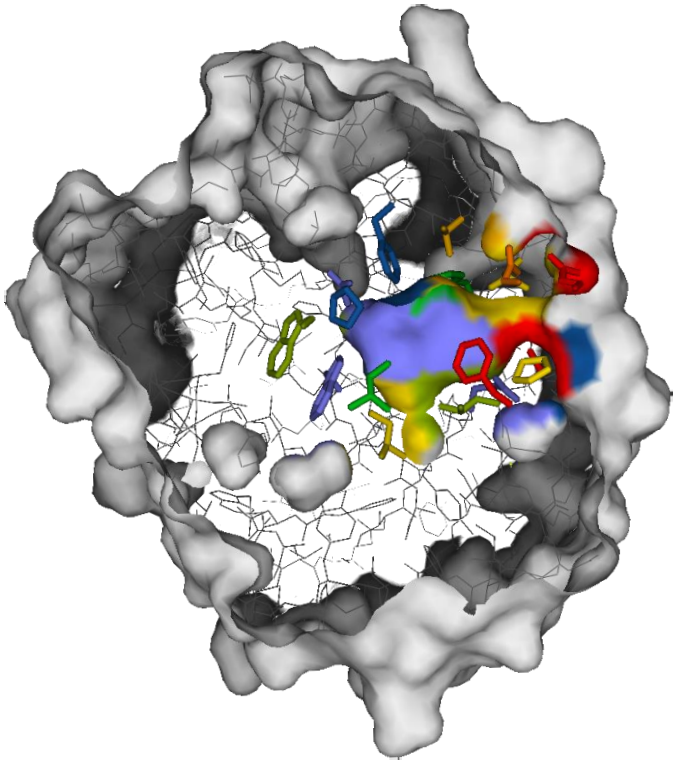
HotSpot Wizard

3. functional residues: active site pocket and tunnels



HotSpot Wizard

4. mutability of individual positions of protein



- Protein engineering approaches
- Semi-rational design
  - identification of hot-spots
  - evaluation of hot-spots
  - selection of substitutions
  - design of library
  - mutagenesis and screening
- Rational design
  - molecular modeling

Design of library

- decisions to be made after evaluation and prioritization of hot-spots:
  - how many and which positions to target?
  - should the positions be randomized simultaneously or separately?
  - should all or only a reduced set of amino acids be introduced at individual positions?

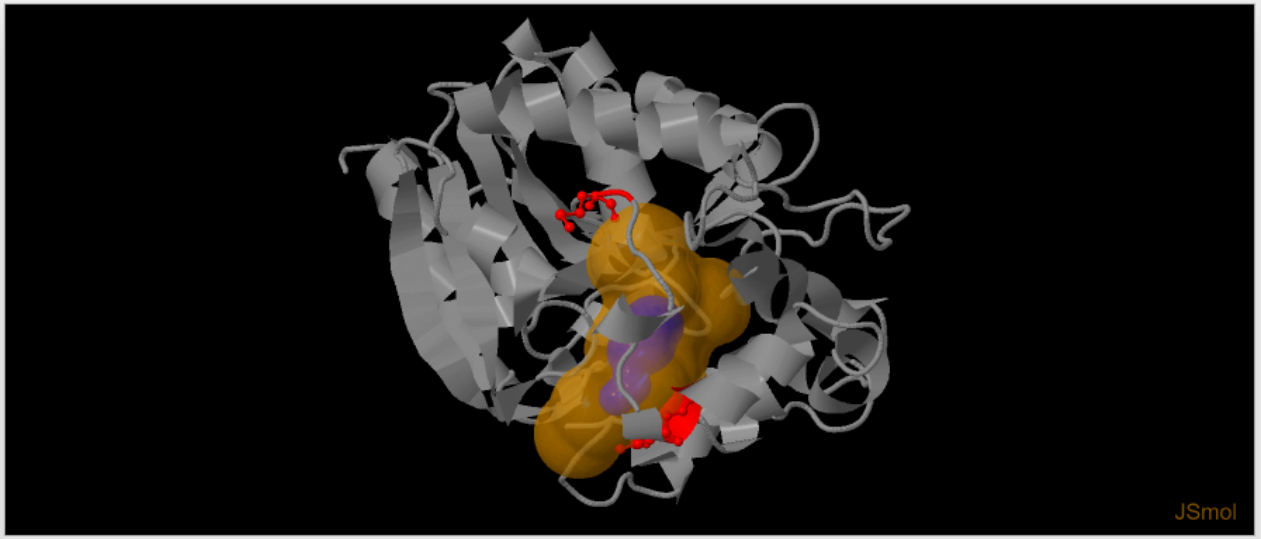
→ dramatic effect on the size of the resulting library

Design of library – HotSpot Wizard

Functional hot spots of 1CV2

Return to Results browser

Viewer



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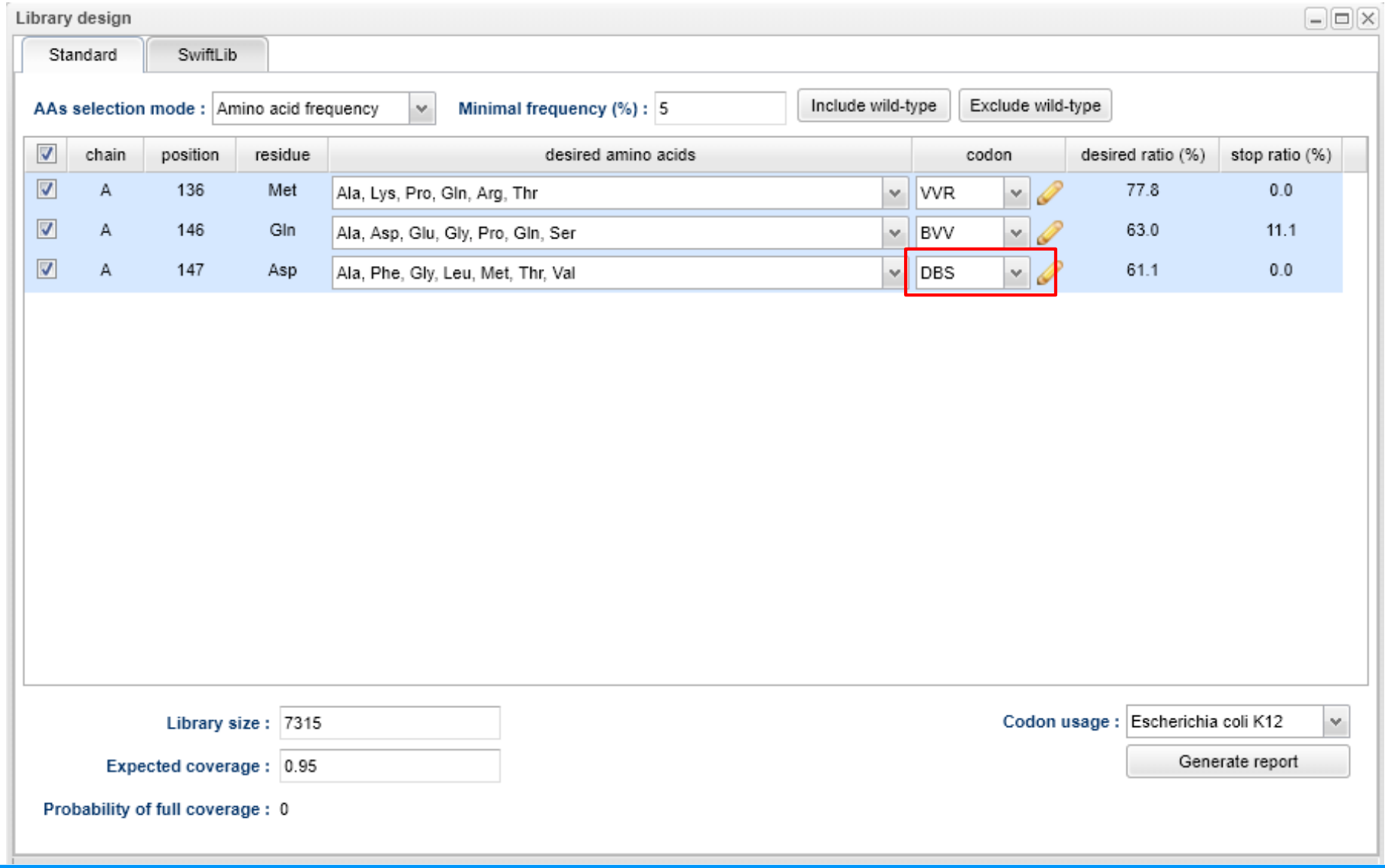
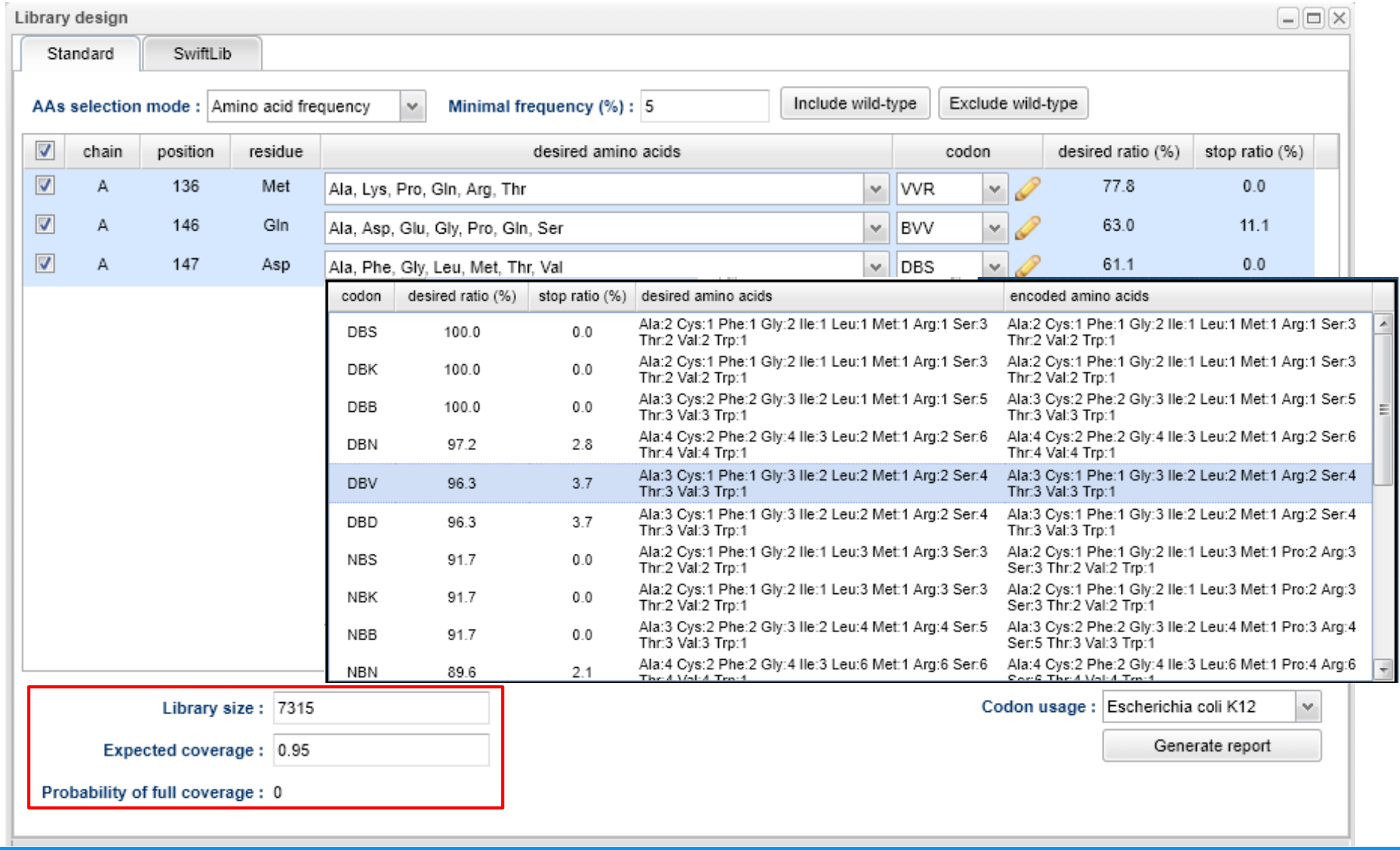
Design of library – HotSpot Wizard



Design of library – HotSpot Wizard



- Protein engineering approaches
- Semi-rational design
  - identification of hot-spots
  - evaluation of hot-spots
  - selection of substitutions
  - design of library
  - mutagenesis and screening
- Rational design
  - molecular modeling

Mutagenesis and screening

- saturation mutagenesis - next lecture ☺

- Protein engineering approaches
- Semi-rational design
  - identification of hot-spots
  - evaluation of hot-spots
  - selection of substitutions
  - design of library
  - mutagenesis and screening
- Rational design
  - molecular modeling→ design of mutations

Rational design

- site-specific changes on the target enzyme
- few amino-acid substitutions that are predicted to elicit desired improvements of enzyme function
- based on detailed knowledge of protein structure, function and catalytic mechanism
- ☺ relatively simple characterization of constructed variants
- ☹ complexity of protein structure-function relationships
- ☹ molecular modeling expertise usually required

- Protein engineering approaches
- Semi-rational design
  - identification of hot-spots
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  - selection of substitutions
  - design of library
  - mutagenesis and screening
- Rational design
  - molecular modeling→ design of mutations

Molecular modeling

- “Theoretical or computational technique that provides insight into the behavior of molecular system.”

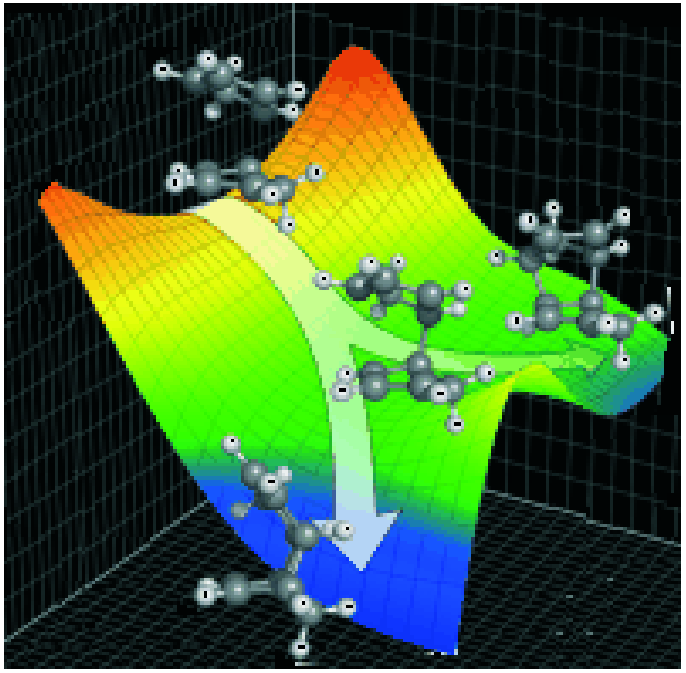
  A. R. Leach

- Applications
  - Protein stabilization
  - prediction of protein dynamics
  - prediction of protein-ligand interactions
  - prediction of reaction barriers and reaction mechanisms

Molecular modeling

- relationship between energy and 3D-structure
  - potential energy surface

- basic methods
  - molecular mechanics
  - molecular dynamics
  - quantum chemistry
  - molecular docking



Design of stability

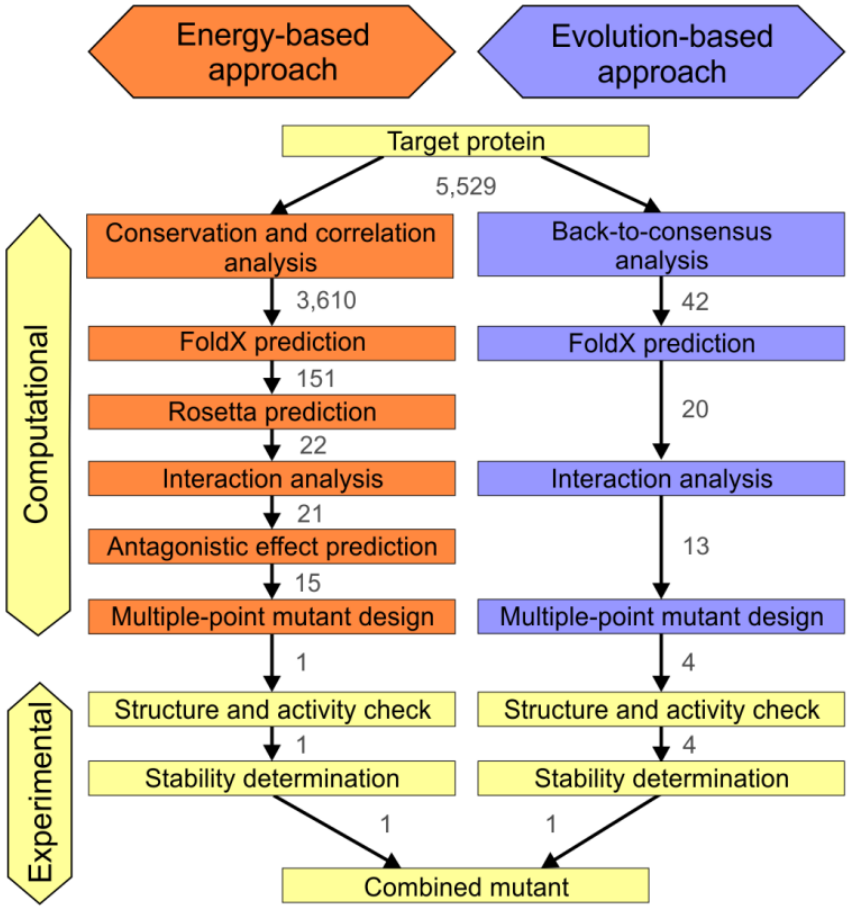
- Enzymes as biocatalysts
  - good activity and selectivity in water solution and standard temperature
  - for many biotechnological applications, high temperature or addition of organic solvents are necessary
  - this conditions can lead to denaturation > importance of stable proteins

Design of stability

- Computational method FireProt

  https://loschmidt.chemi.muni.cz/fireprot/

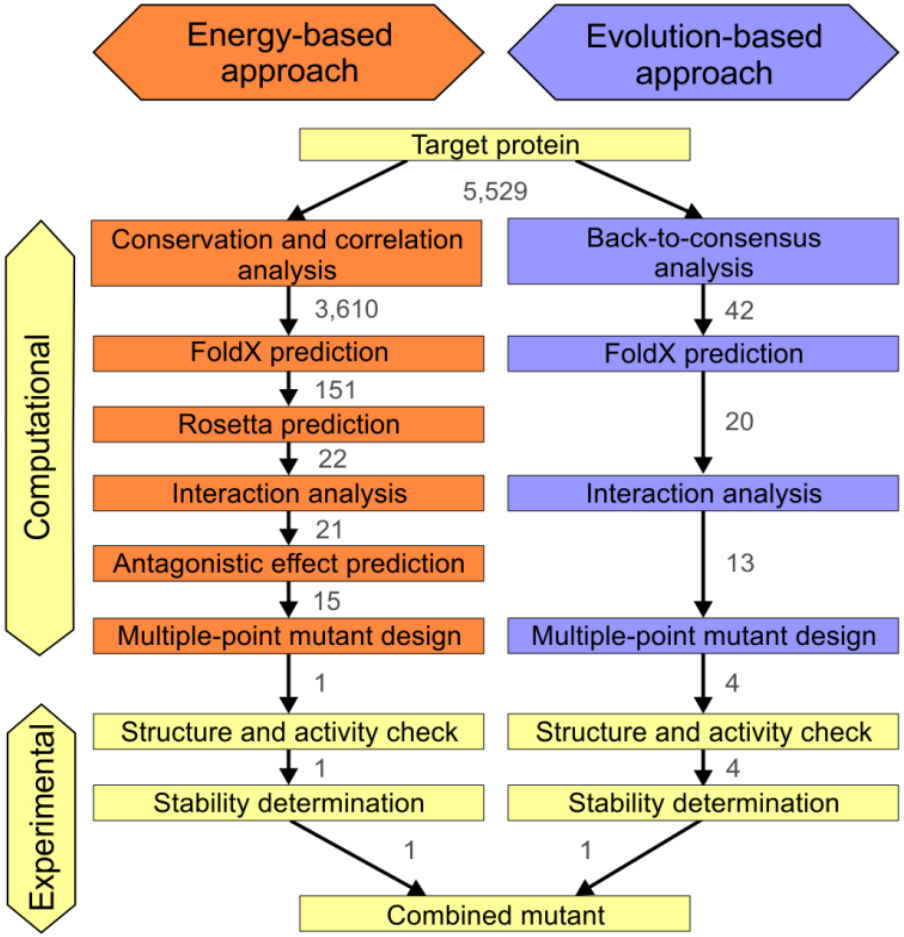
  - prediction of all single-point mutants by FoldX, Rosetta, and back-to-consens
  - smart filtering based on conservation, correlation, electrostatic interactions, and antagonistic effect
  - final prediction of multiple-point mutants for gene synthesis



Design of stability

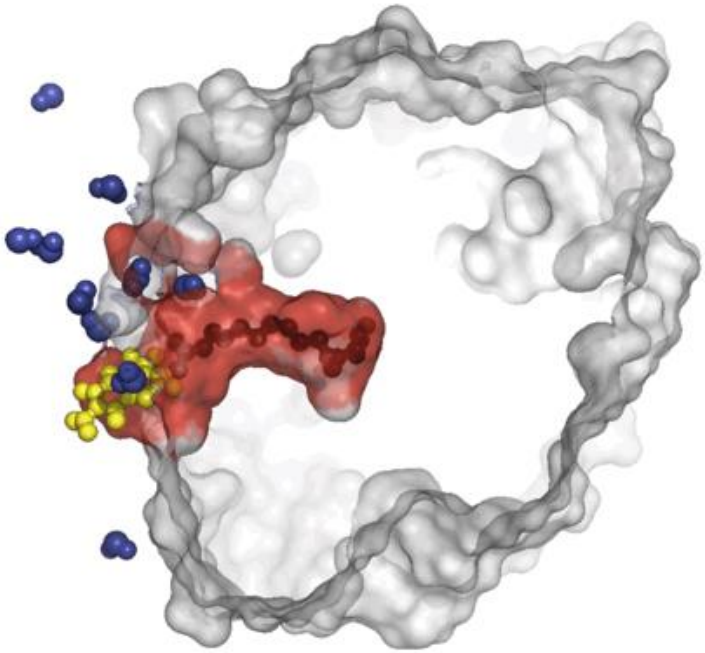
Racionální design stabilnějších enzymů

- Stabilization of haloalkane dehalogenase DhaA
  - *In silico* prediction of 5,500 mutants
  - Experimental testing of 5 mutants
- Output
  - 3 more stable mutants
  - Combined mutant ΔT_m = 24°C



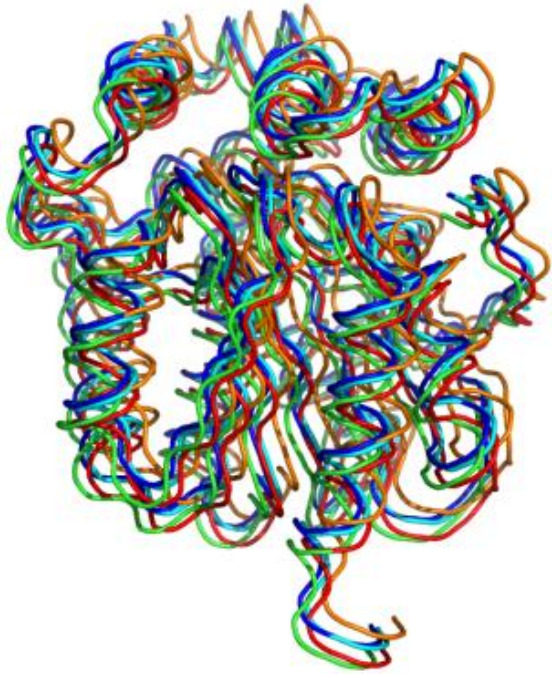
Molecular dynamics

- successive configurations of system in time
- provides information on energetics, amplitudes and time scales of local motions on atomic level



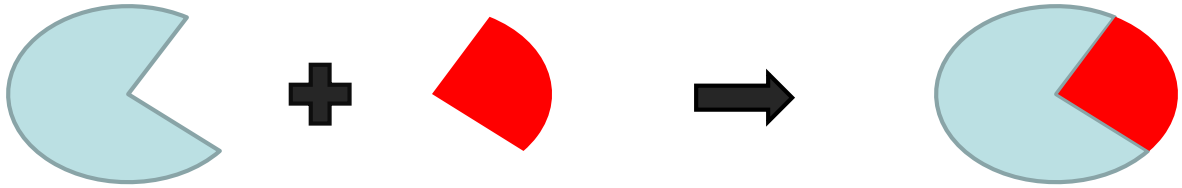
Molecular dynamics

- generates ensemble of structures
  - more precise calculations of free energies



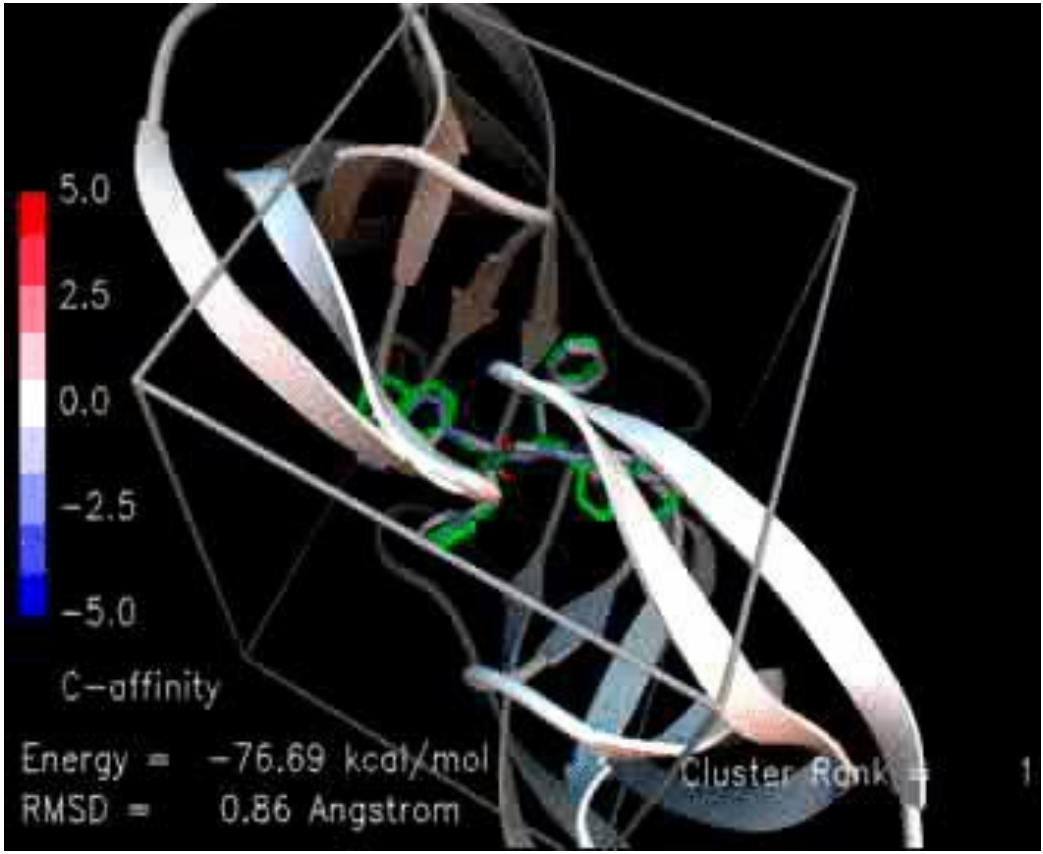
Molecular docking

- predicts structure of receptor (protein) – ligand complex



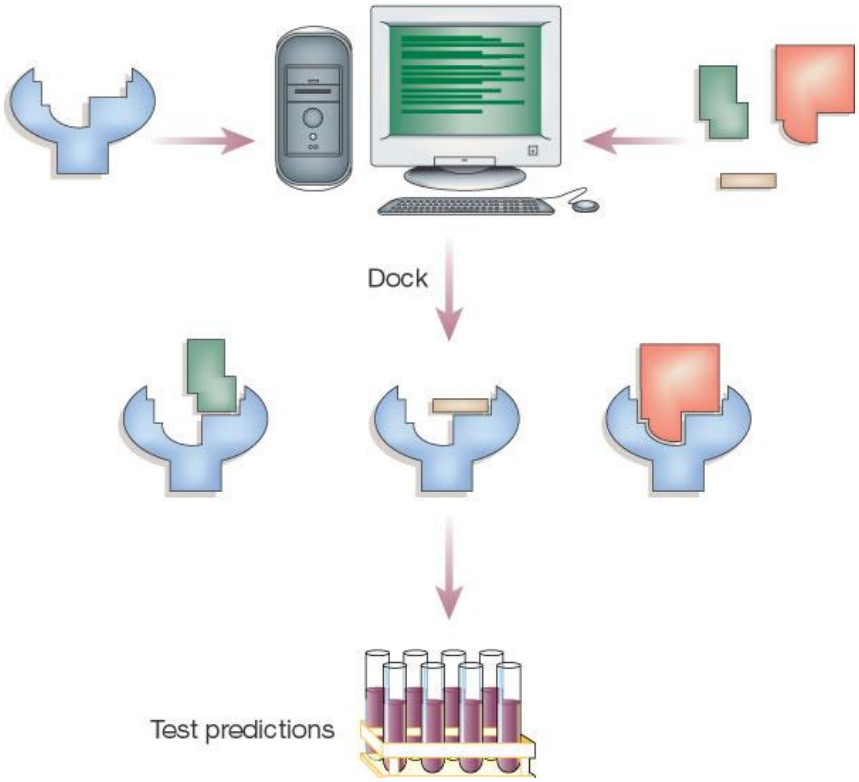
Molecular docking

- Two components procedure
  - searching – finding the conformation of ligand in the active site of the enzyme
  - scoring – evaluation of the binding free energy
- Docking software
  - Autodock, Vina, Gold, Medusa, Rosetta Dock…



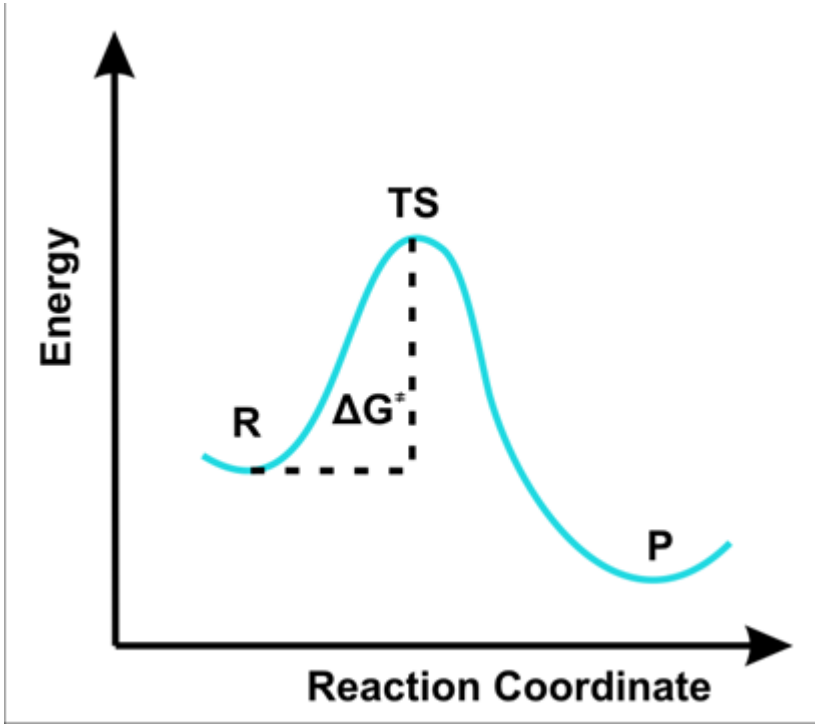
Molecular docking

- Virtual screening
  - many compounds against one enzyme
  - one compound against many enzymes



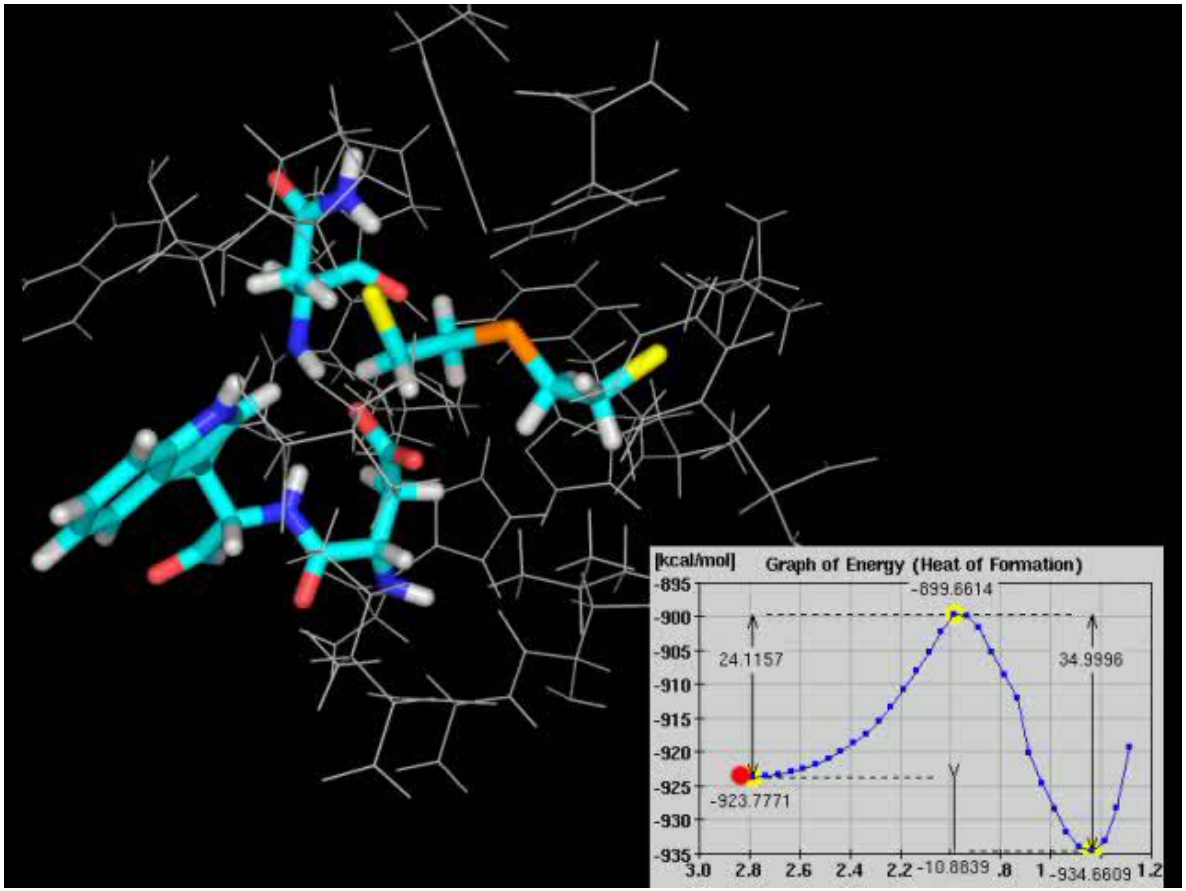
Quantum chemistry

- modeling of reaction
  - reaction barrier



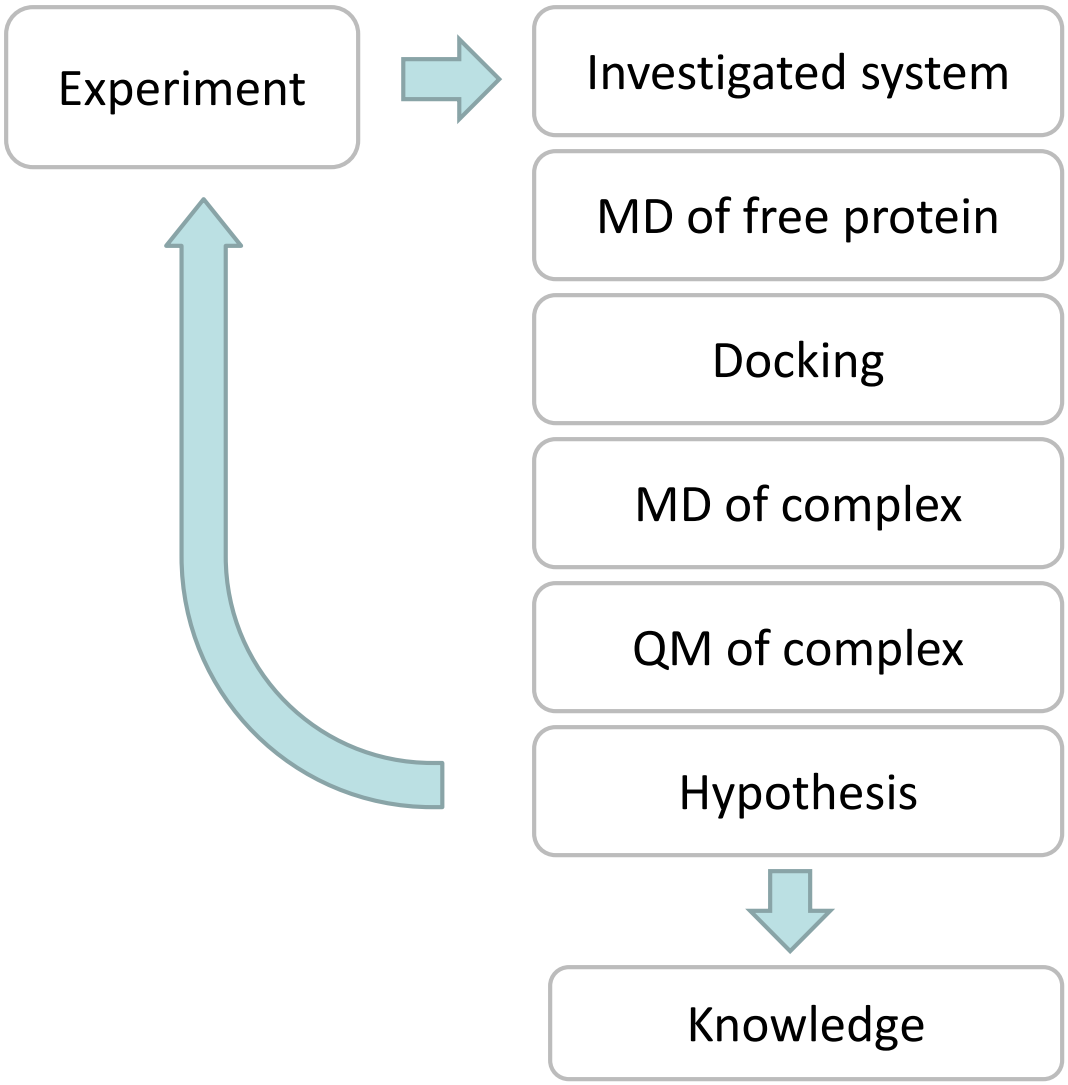
Quantum chemistry

- modeling of reaction



TRITON

Design of mutations

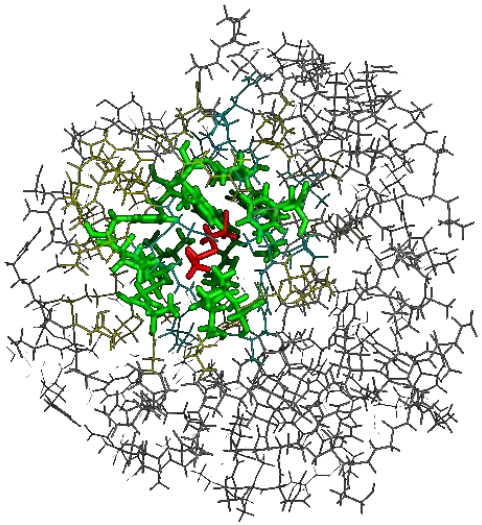


Design of mutations

- identification of functionally important residues
  - decomposition of energies to individual contribution
  - flexible residues – functionally important dynamics
  - residues in contact with ligand

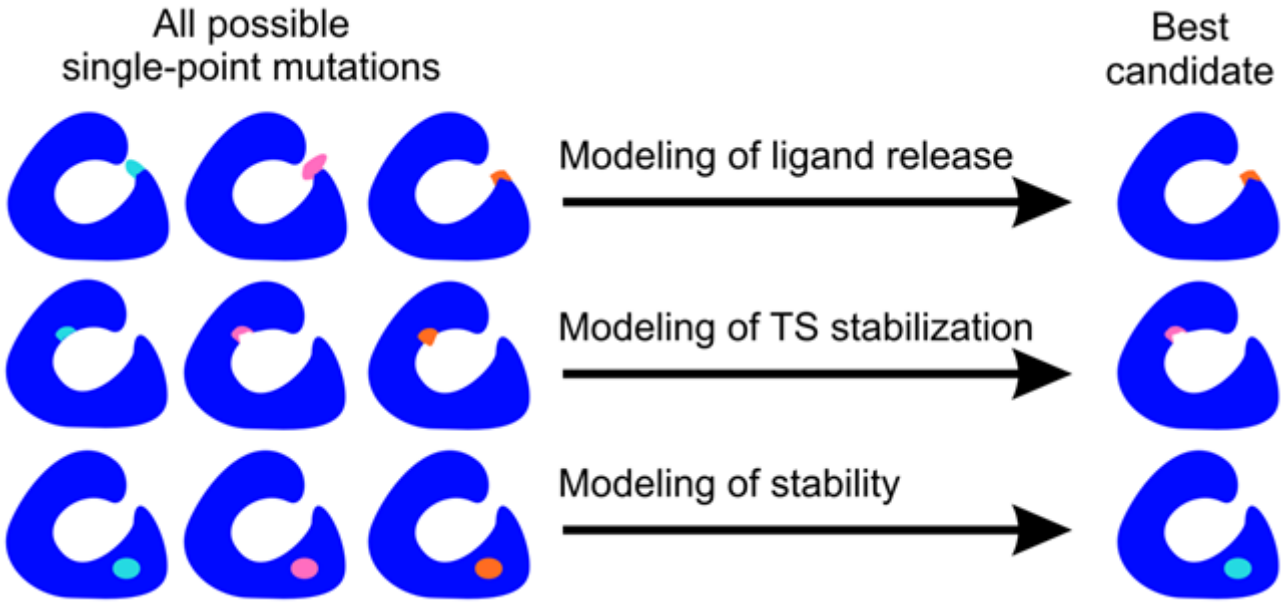
→ further molecular modeling

→ semi-rational design



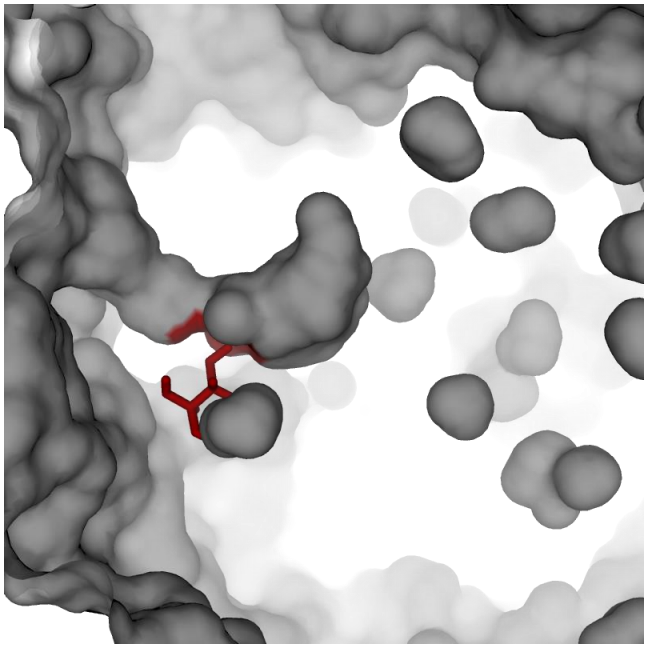
Design of mutations

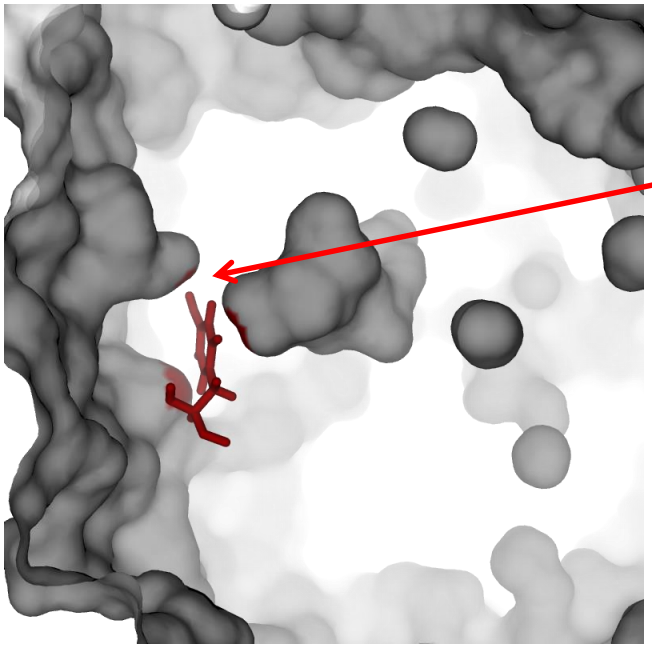
- design of modified enzymes by *in silico* screening
  - study of effects of all relevant mutations
  - selection and combination of the best mutations



Design of mutations

- effect of mutations at molecular level
  - example: improved activity of tunnel mutant





closed tunnel

+

improved activity

LOSCHMIDT LABORATORIES

PROTEIN ENGINEERING

8. Directed evolution

Loschmidt Laboratories
Department of Experimental Biology
Masaryk University, Brno