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

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Protein engineering

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

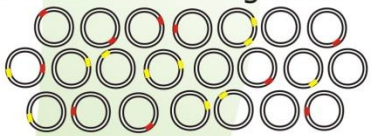
6. *not applied*

Constructed mutant enzyme

DIRECTED EVOLUTION

1. *not applied*

2. Random mutagenesis



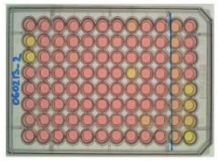
Library of mutated genes
(>10,000 clones)

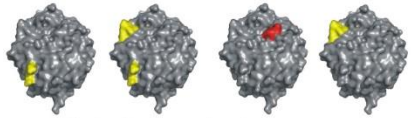
3. Transformation

4. Protein expression

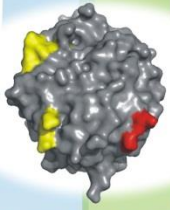
5. *not applied*

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

Structure information

- **worldwide Protein Data Bank (wwPDB)**
  - http://www.wwpdb.org/
  - central repository of ~180,000 experimental macromolecular structures (April 2021)
- **RCSB PDB**
  - https://www.rcsb.org/
- **PDBe**
  - https://www.ebi.ac.uk/pdbe/
- **PDBj**
  - https://pdbj.org/

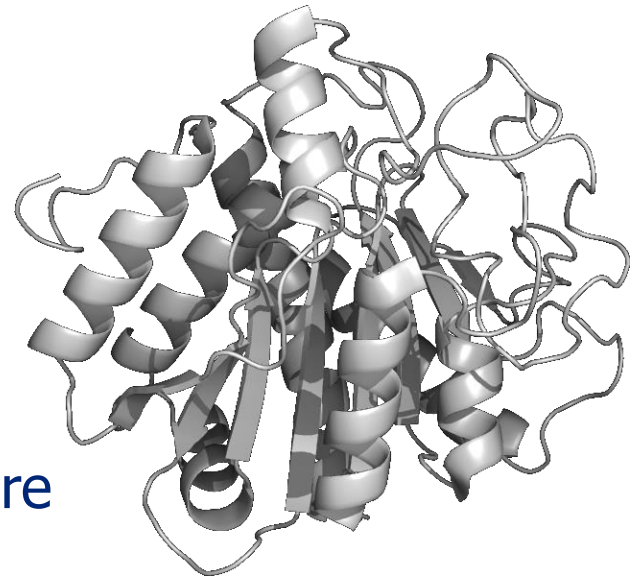
Homology modelling

3D structure is determined by the sequence

MSLGAKPFGEKKFIEIKGRRMAYIDEGTGDPILFQHGNPTSSYLWRNI
MPHCAGLGRLIACDLIGMGDSDKLDPSGPERYAYAEHRDYLDALWEA
LDLGDRVVLVVHDWGSALGFDWARRHRERVQGIAYMEAIAMPIEWA
DFPEQDRDLFQAFRSQAGEELVLQD

sequence

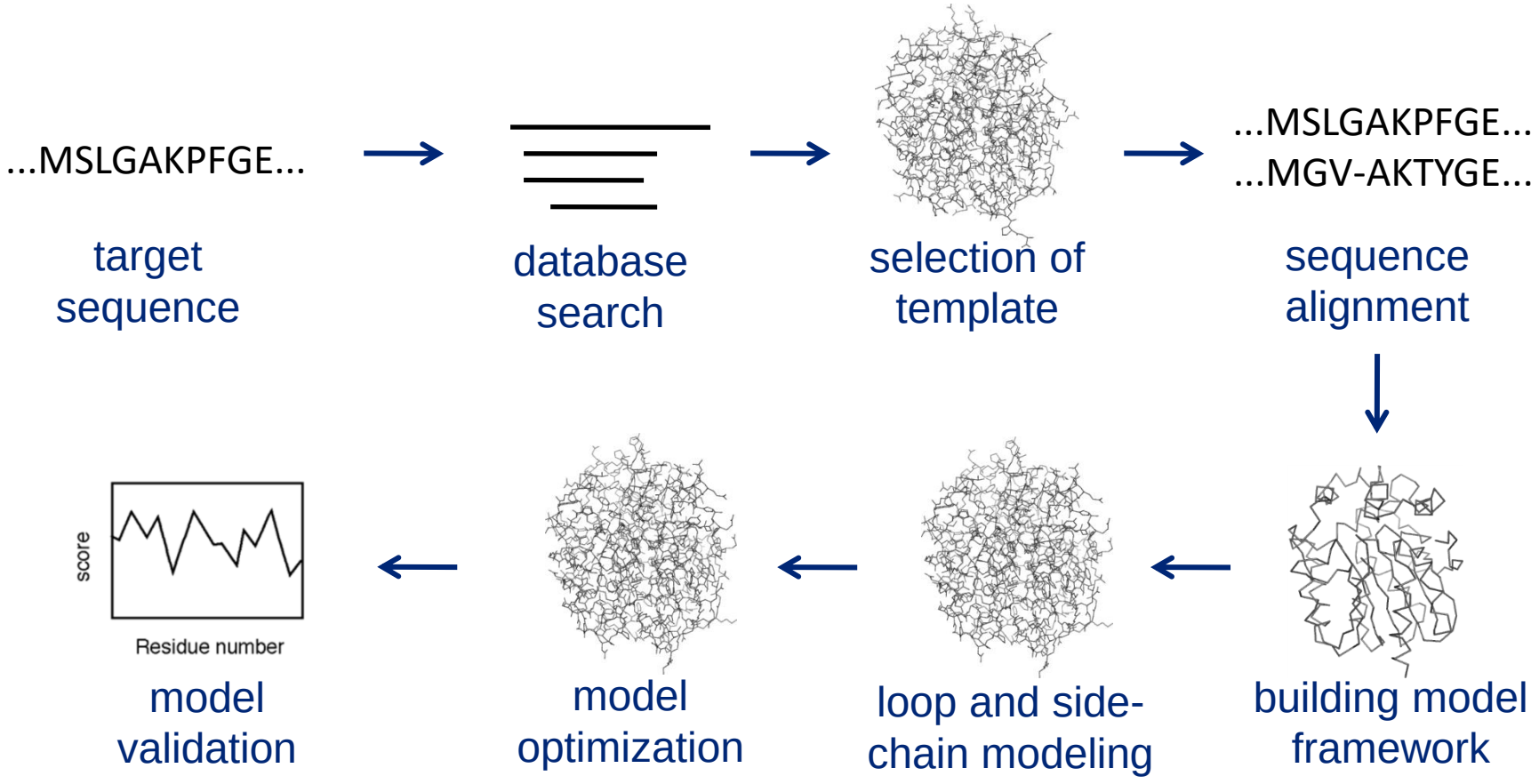
prediction



structure

function

Homology modelling



Homology modelling - tools

- **MODELLER**
  - http://salilab.org/modeller/
- **SWISS-MODEL**
  - http://swissmodel.expasy.org/
- **Robetta**
  - http://robetta.bakerlab.org/
- **I-TASSER**
  - https://zhanglab.ccmb.med.umich.edu/I-TASSER/

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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

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- Rational design
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Identification of hot-spots

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

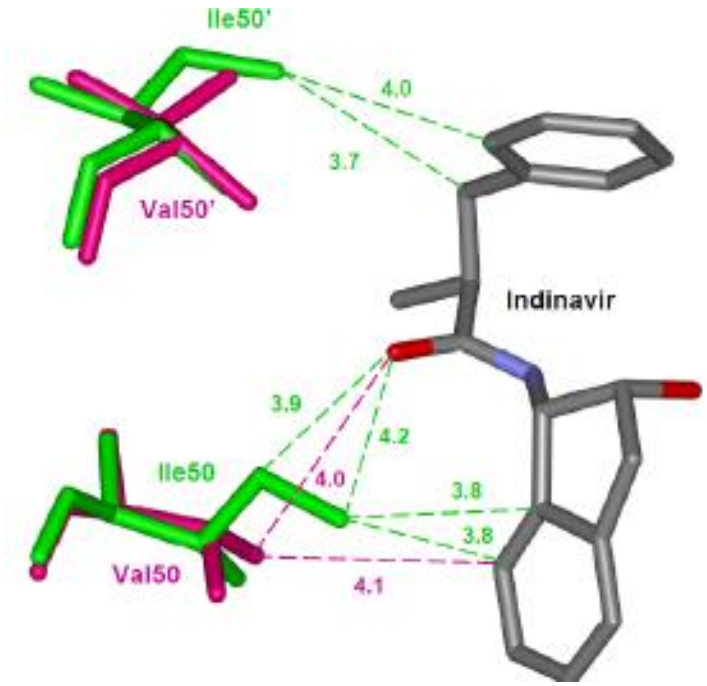
Hot-spots for engineering catalytic properties

- 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!

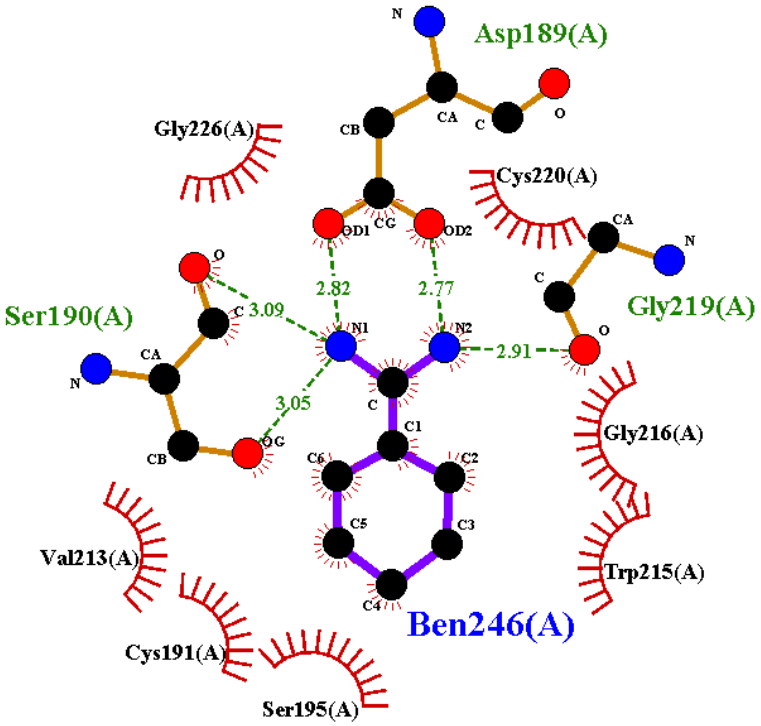
Analysis of protein-ligand interactions

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

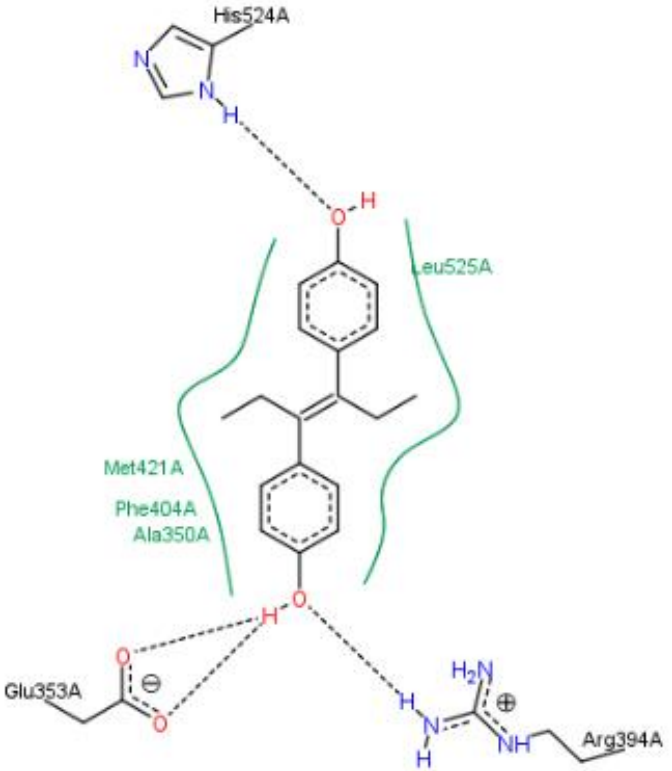


Analysis of protein-ligand interactions

- schematic diagrams of protein-ligand interactions



LigPlot, LigPlot+

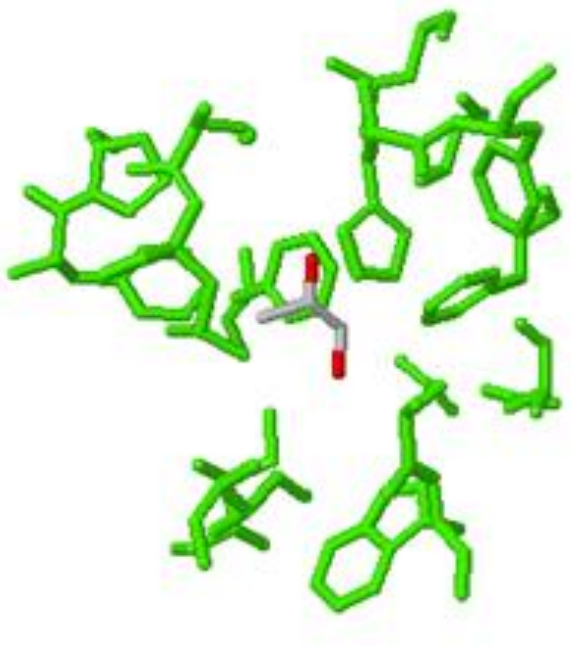


PoseView

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

Essential residues

UniProt (Swiss-Prot) - https://www.uniprot.org/

Sites

Feature key	Position(s)	Description
Binding site	38	Halide
Active site	108	Nucleophile 3 Publications
Binding site	109	Halide
Active site	132	Proton donor 3 Publications
Active site	272	Proton acceptor 3 Publications

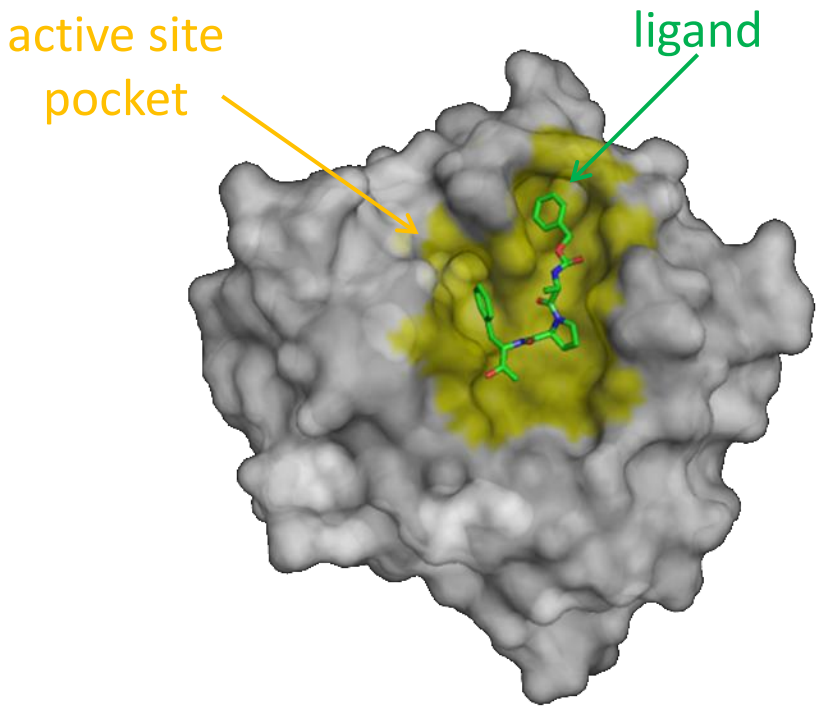
Catalytic Site Atlas - https://www.ebi.ac.uk/thornton-srv/m-csa/

Catalytic Residues Roles

UniProt	PDB*	
His272	His272A	Acts as general acid base to deprotonate water, thus activating water so its lone pair can attack the covalent enzyme intermediate.
Asp108	Asp108A	Acts as nucleophile on the electrophilic carbon atom to form a covalent enzyme intermediate which is hydrolysed to give the product.
Asn38, Trp109	Asn38A, Trp109A	Involved in stabilisation of the halogen, transition-states and product.
Glu132	Glu132A	Acts to modify the pKa of His 272 so that it remains in the correct protonation state for its role in catalysis.

Analysis of binding pockets

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



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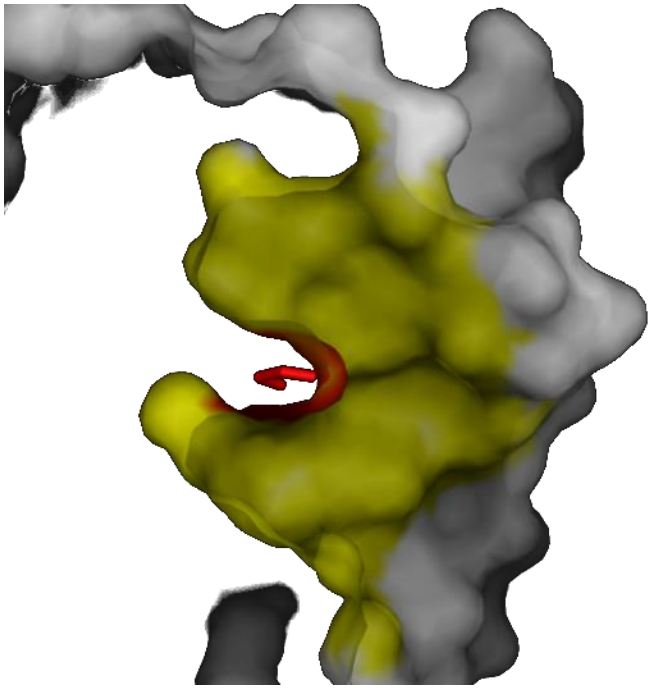

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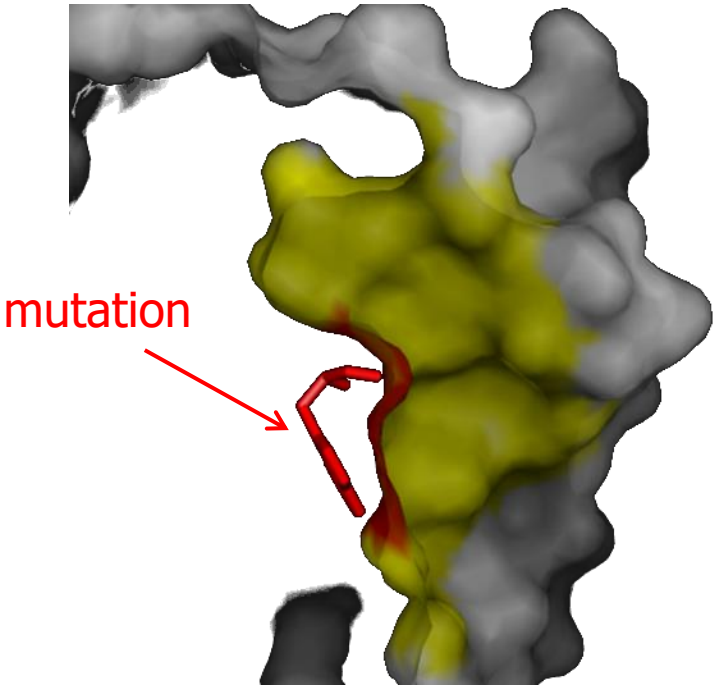

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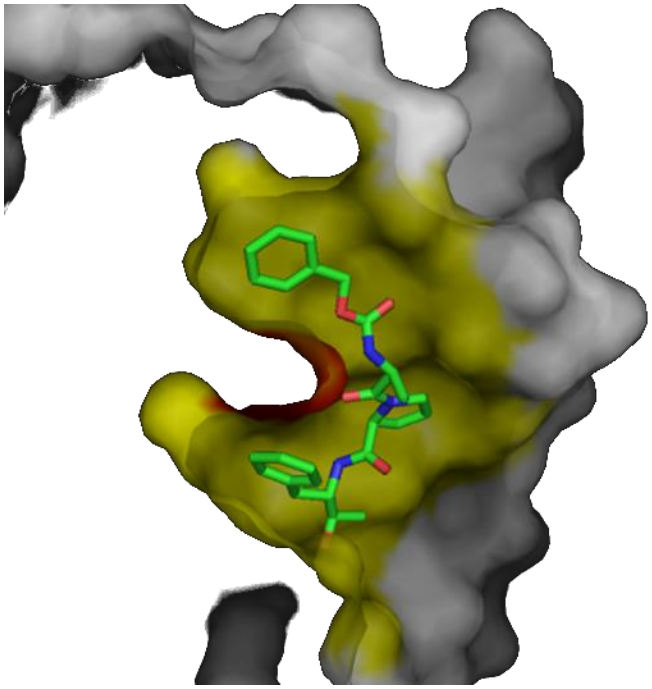

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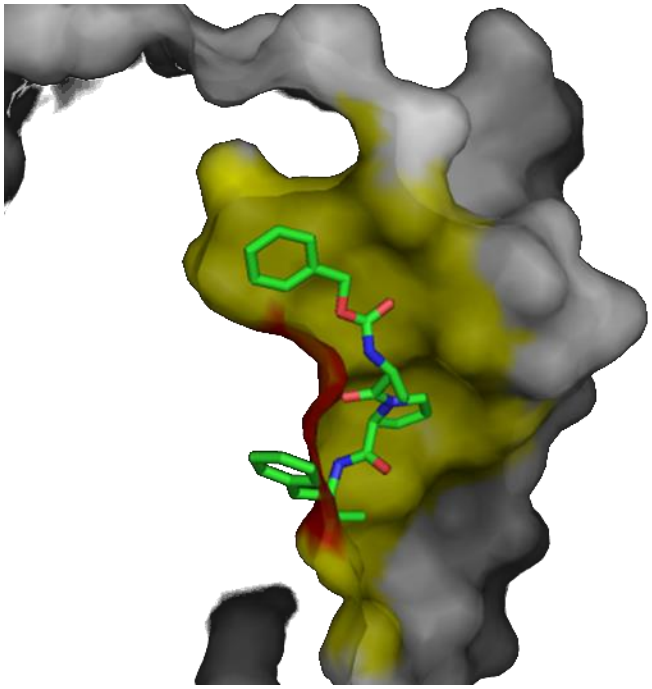
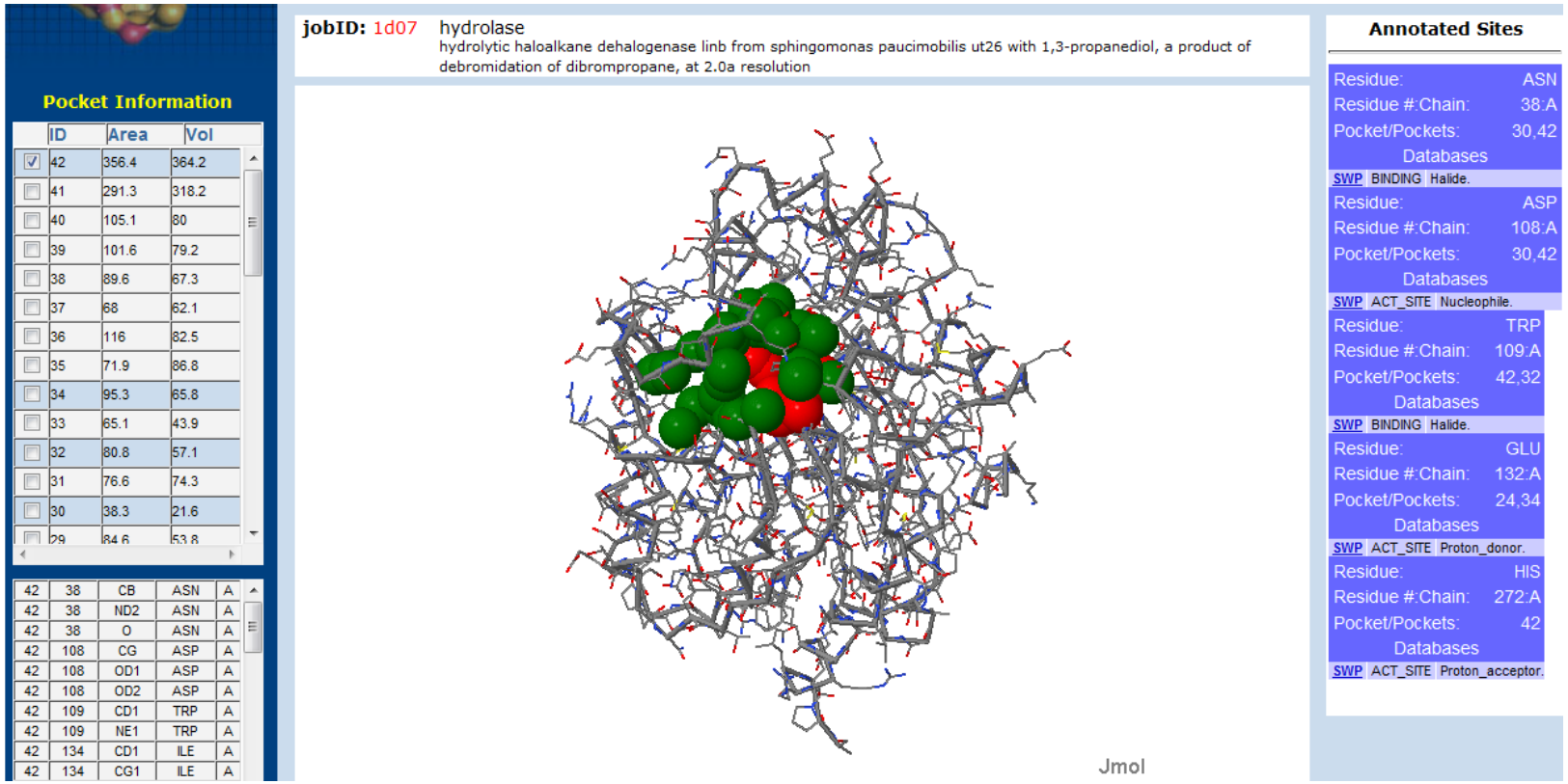


Analysis of binding pockets

- 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

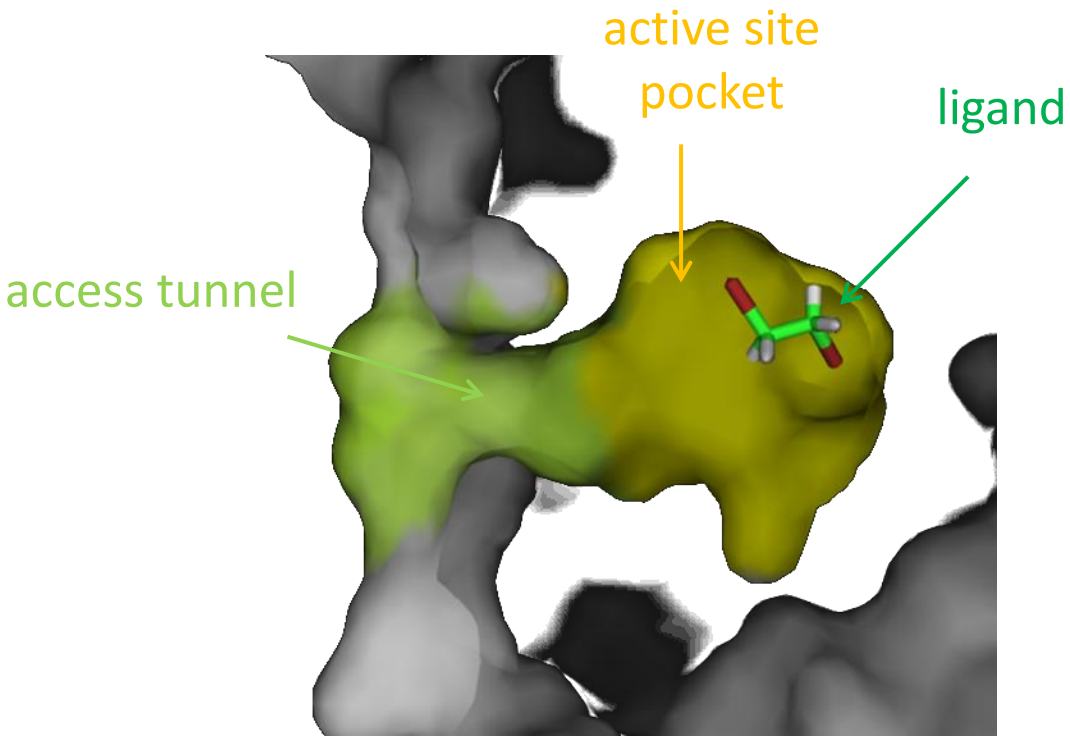
- detailed characterization of all pockets in the structure



CASTp

Analysis of access tunnels

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



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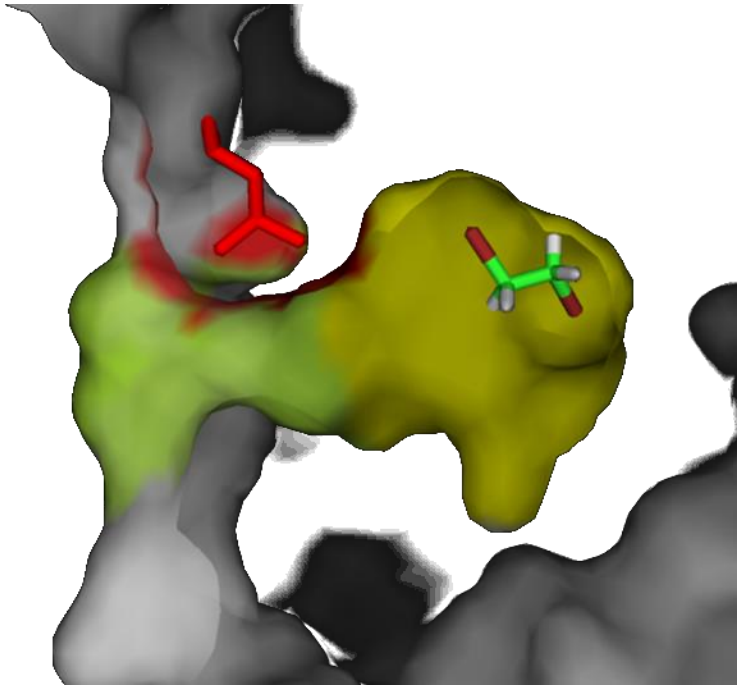

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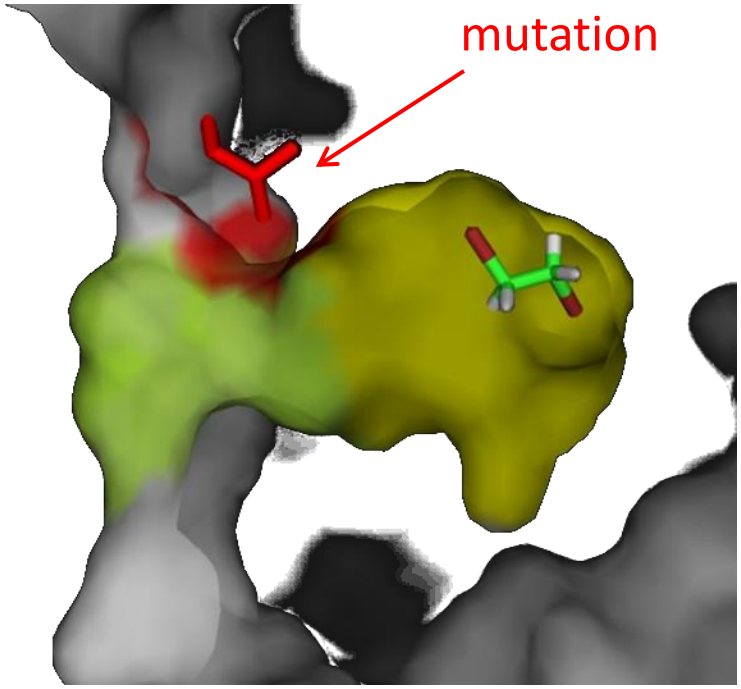

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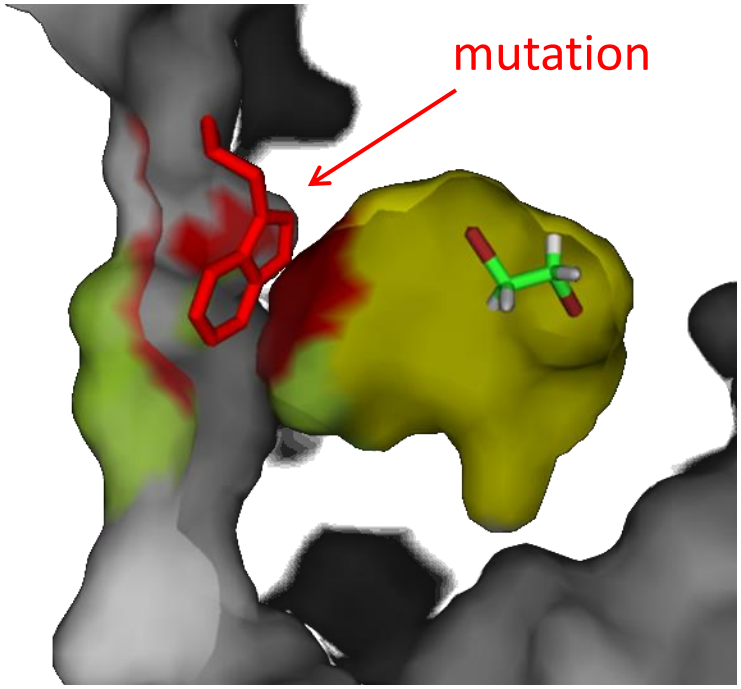


Analysis of access tunnels

- 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

- Detection and detailed characteristics of access tunnels

CAVER Analyst 2.0

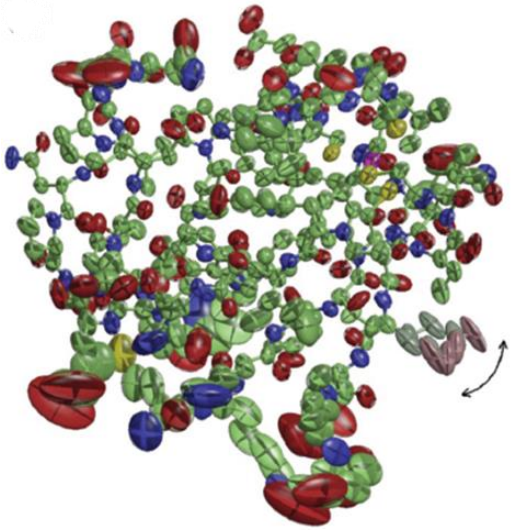
Hot-spots for engineering thermostability

- 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

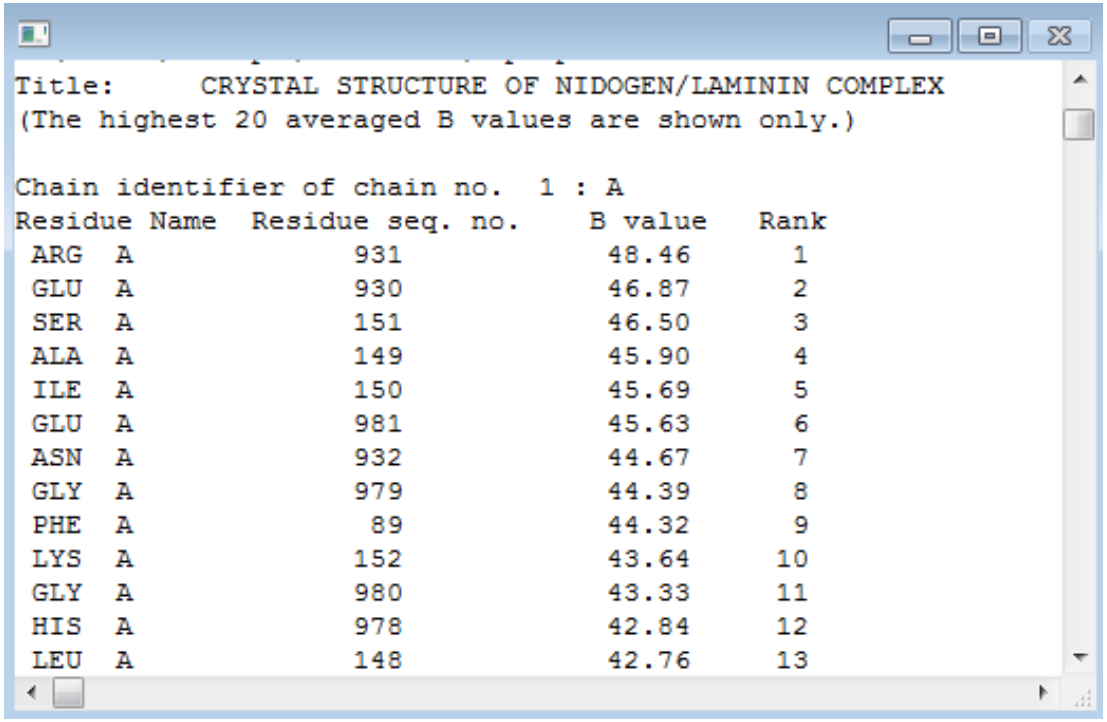
- prediction based on crystallographic B-factors
  - reflect the degree of thermal motion, and thus the flexibility of individual residues



- 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.)

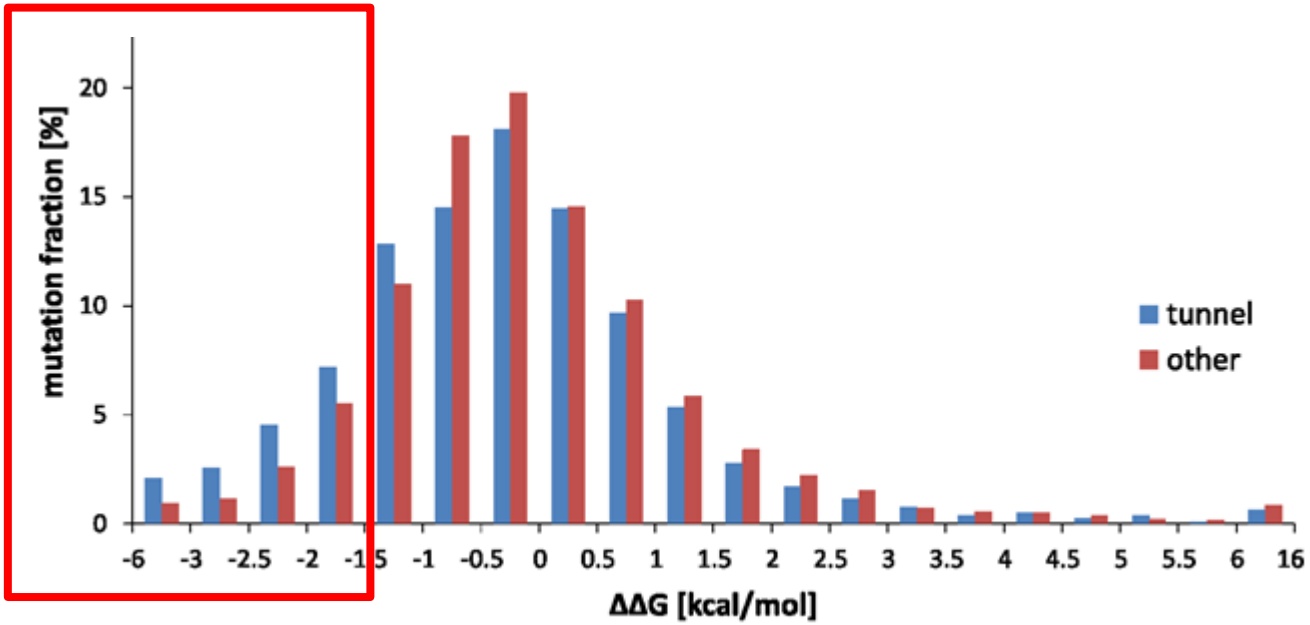
Chain identifier of chain no. 1 : A

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

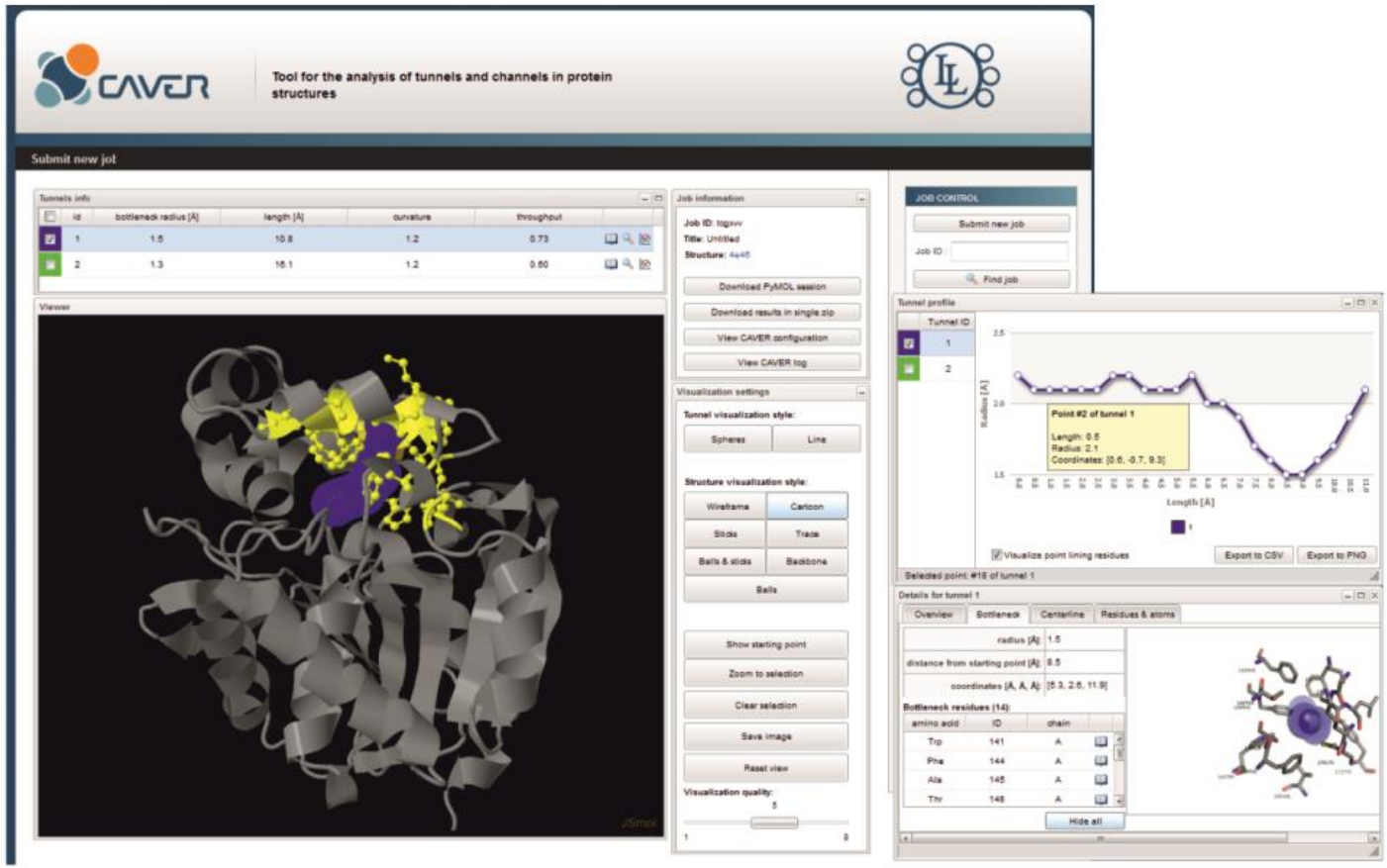
Analysis of access tunnels

- 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



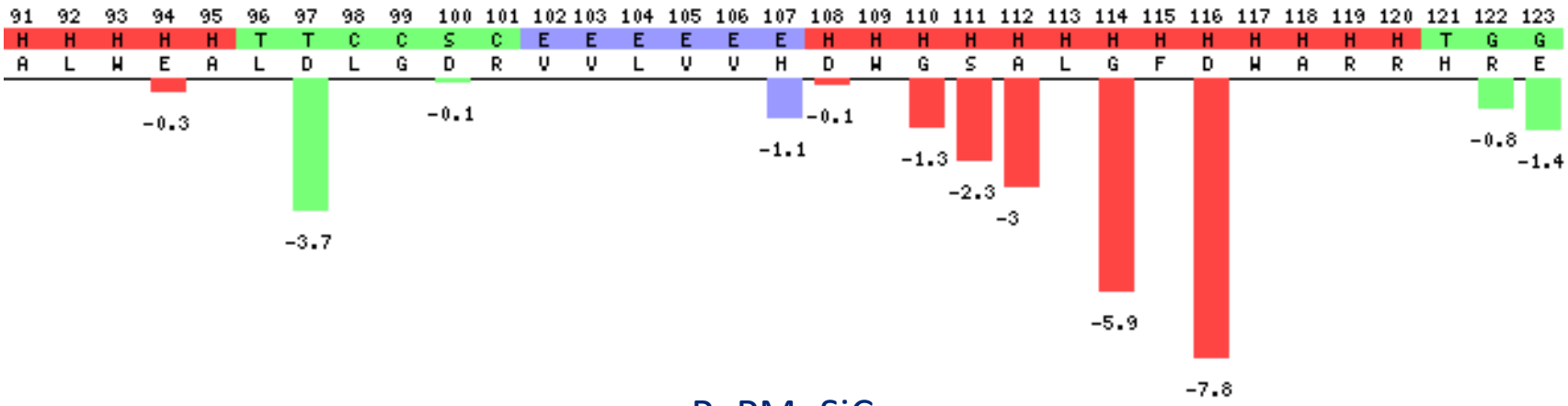
CAVER Web

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)

Analysis of evolutionary conservation

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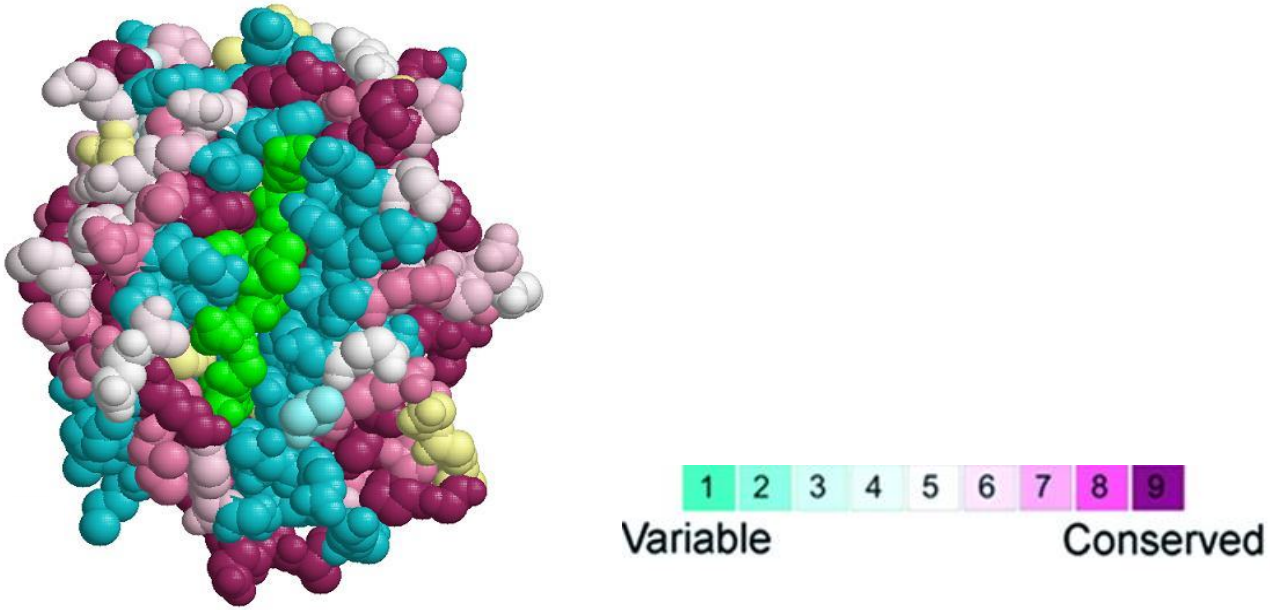
Analysis of evolutionary conservation

- 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

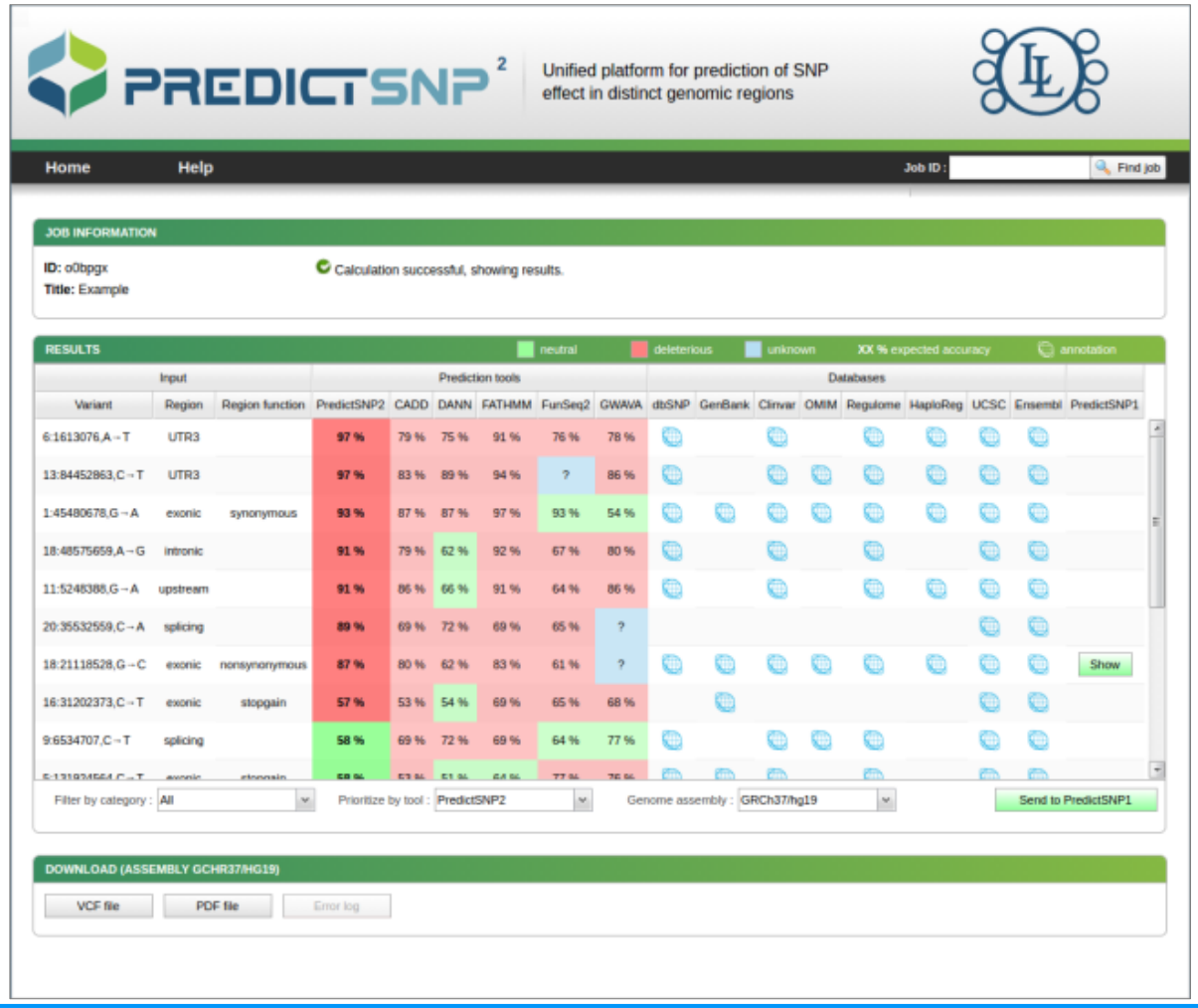
CUPSAT

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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  - identification of hot-spots
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  - selection of substitutions
  - design of library
  - mutagenesis and screening
- Rational design
  - molecular modeling

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

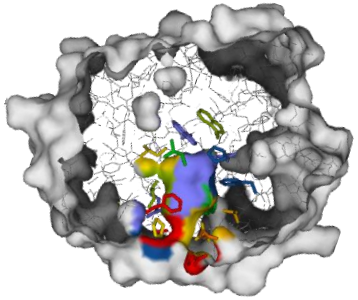
- introduction of amino acids exhibiting certain properties
- introduction of a balanced set of amino acids
- introduction of substitutions existing (at a given site) in known natural proteins
- discarding amino acids with potentially destabilizing/ deleterious effects
  - can be obtained by prediction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

HotSpot Wizard

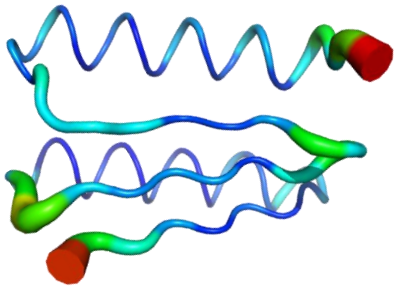
- meta-server combining several tools
  - automatic identification of hot-spots for engineering of enzyme catalytic properties
  - prioritization of hot-spots by their mutability
  - distribution of amino acids at individual positions

HotSpot Wizard

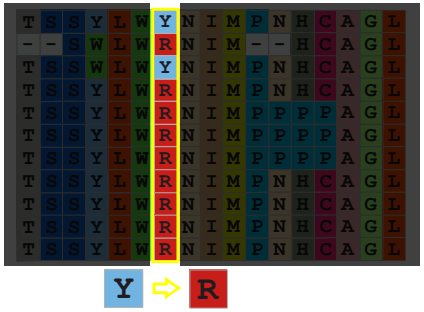
Functional hot-spots



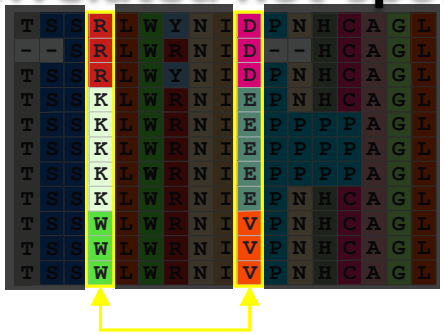
Stability hot-spots (flexibility)



Stability hot-spots (evolution)



Correlated hot-spots

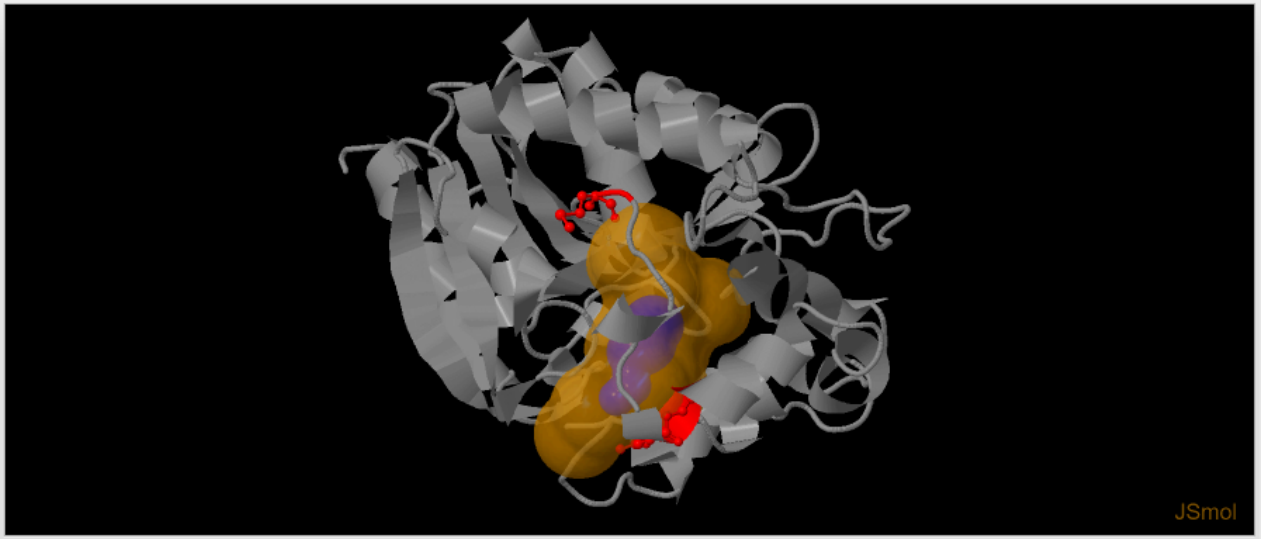


HotSpot Wizard

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
A	146	Gln	✓
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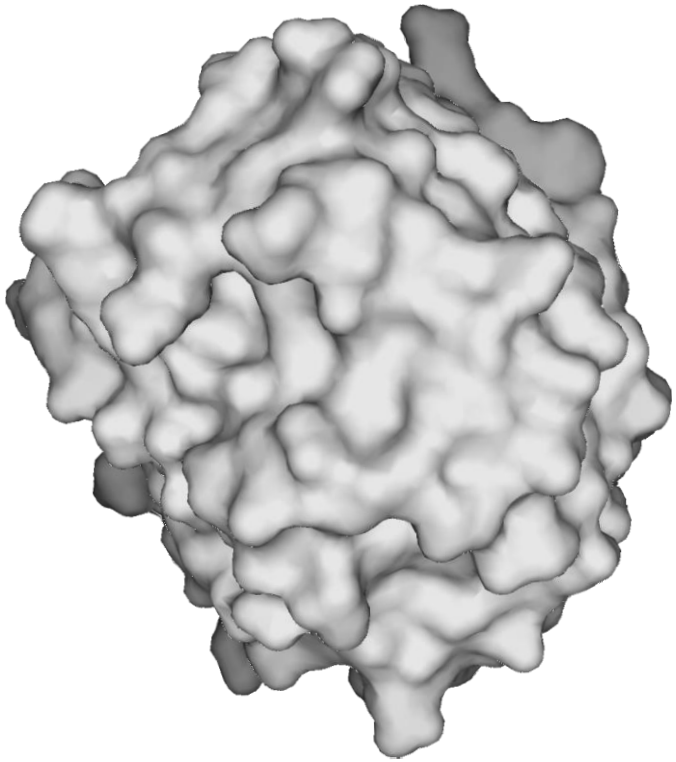
A	147	Asp	✓

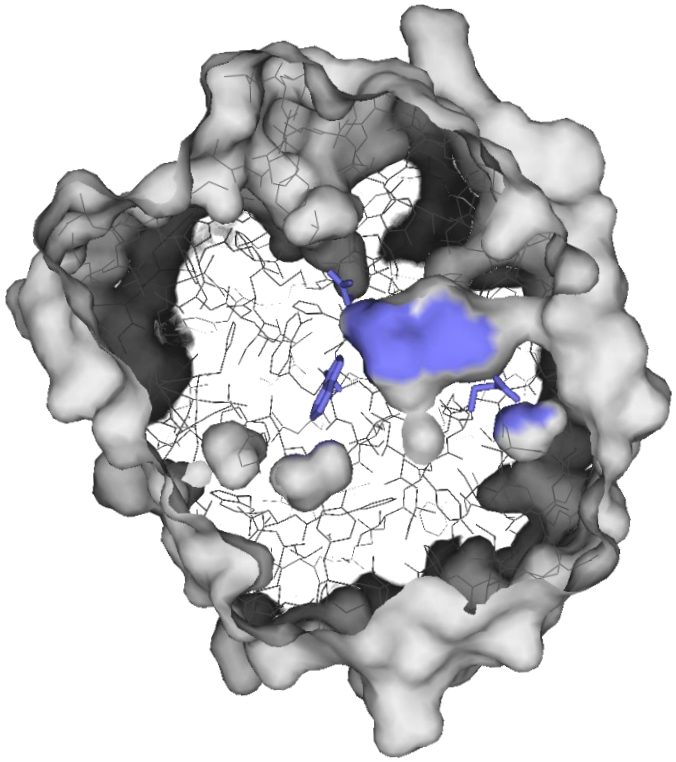
HotSpot Wizard

1. protein structure



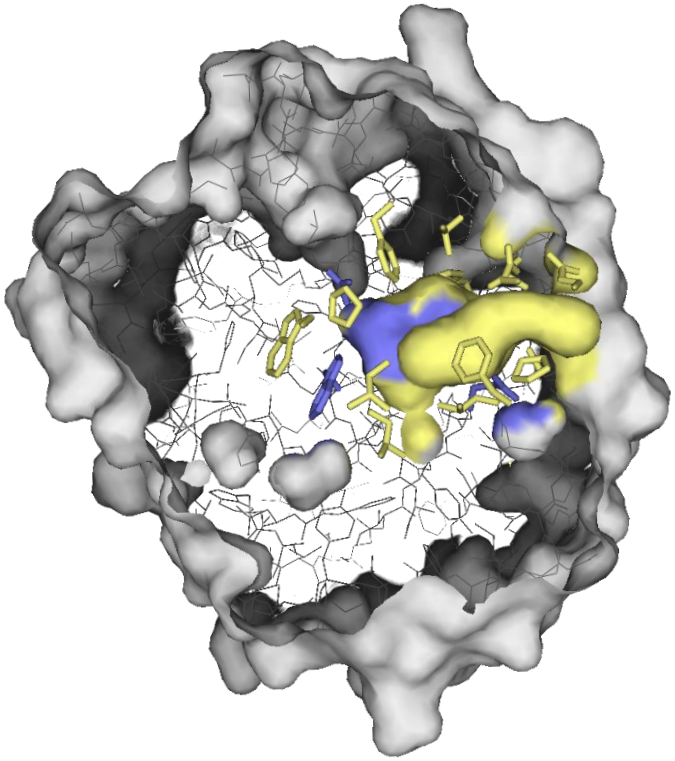
HotSpot Wizard

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



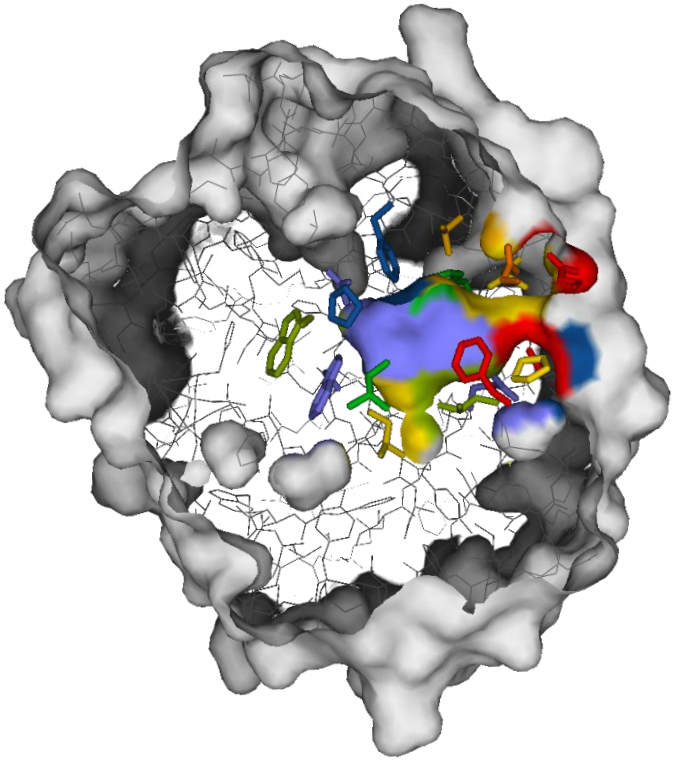
HotSpot Wizard

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?

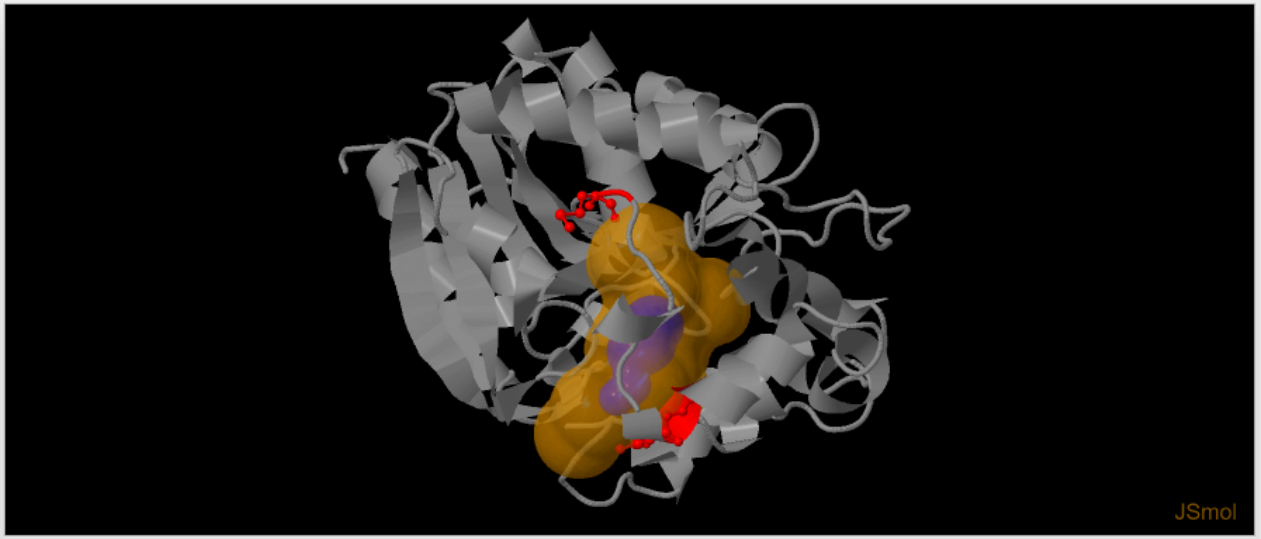
→ dramatic effect on the size of the resulting library

Design of library – HotSpot Wizard

Functional hot spots of 1CV2

Return to Results browser

Viewer



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Residues selected for mutagenesis

Zoom residues | Design library

	chain	position	residue	HotSpot
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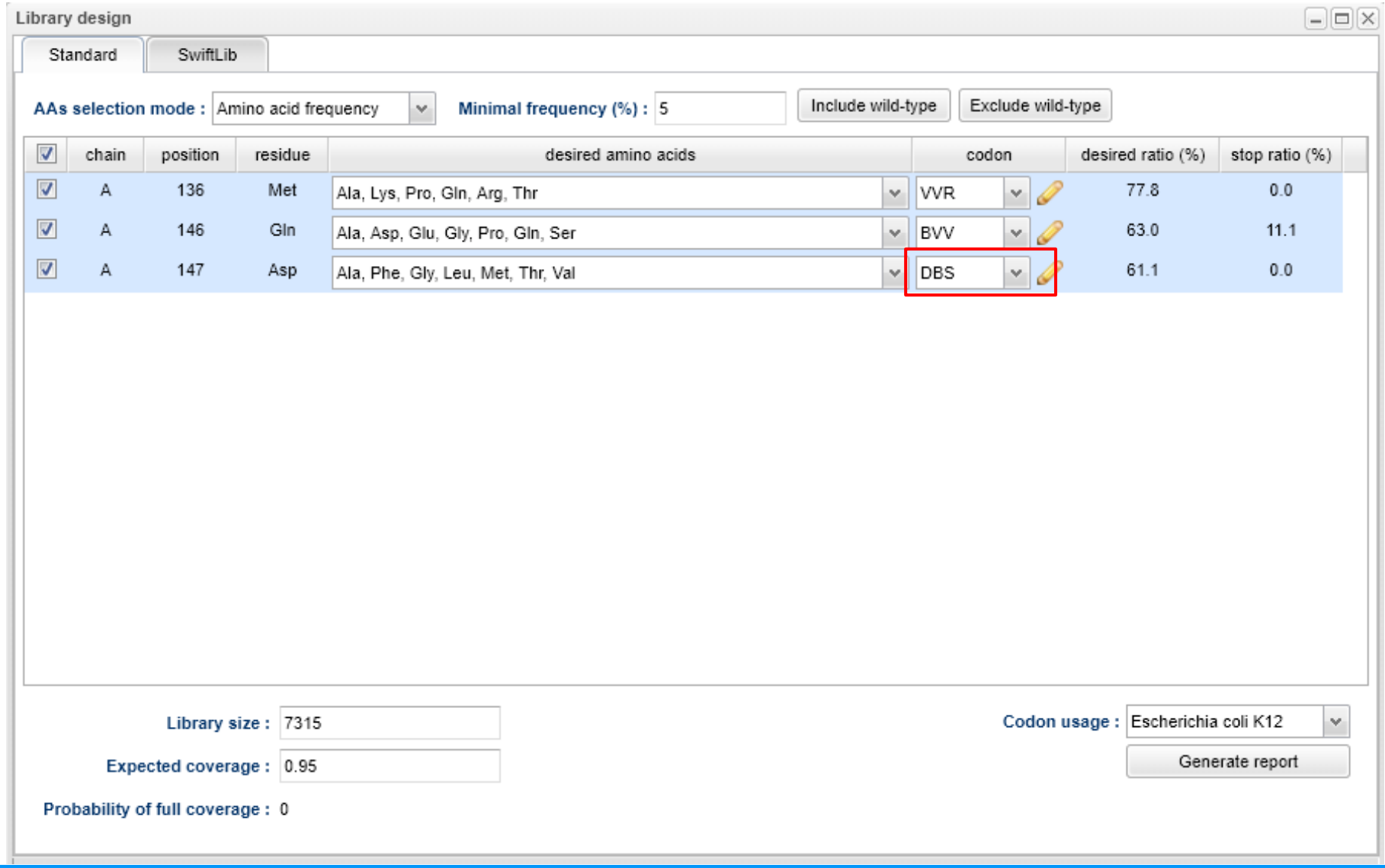
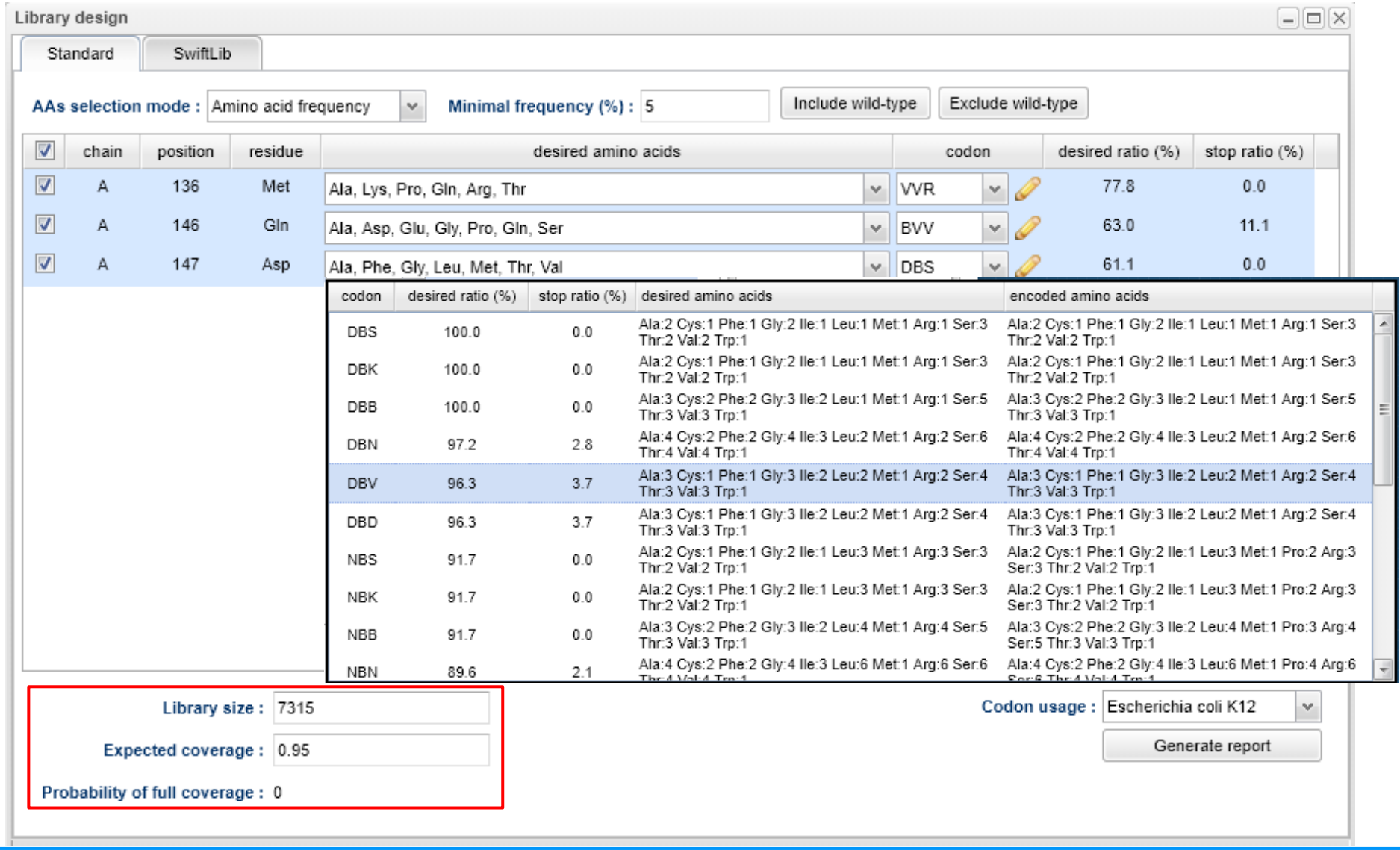
Design of library – HotSpot Wizard



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
  - evaluation of hot-spots
  - 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.”

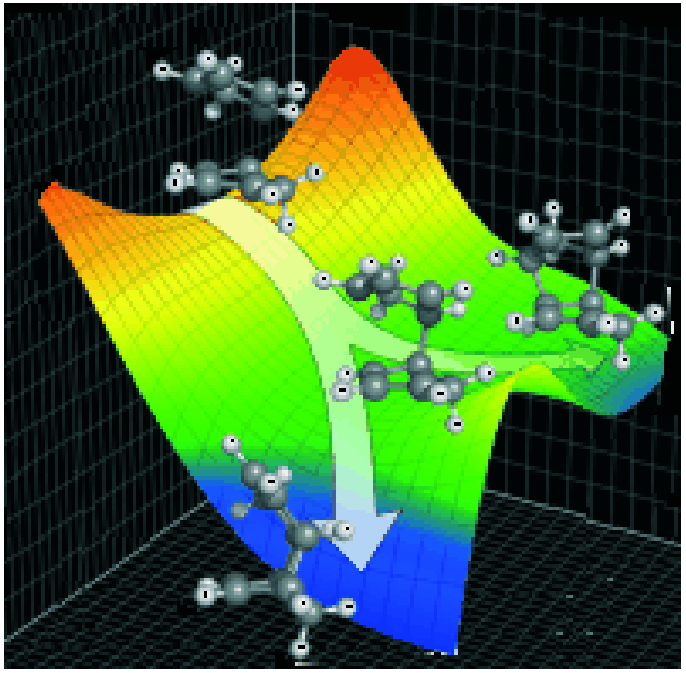
  A. R. Leach

- 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

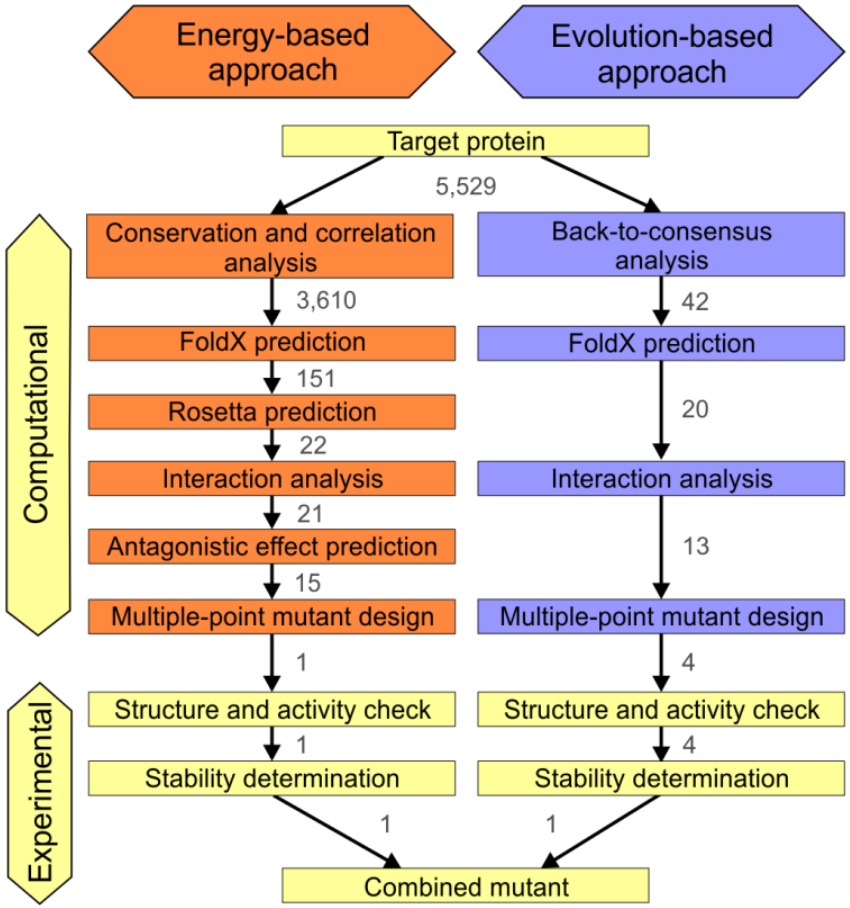
- Enzymes as biocatalysts
  - good activity and selectivity in water solution and standard temperature
  - for many biotechnological applications, high temperature or addition of organic solvents are necessary
  - this conditions can lead to denaturation > importance of stable proteins

Design of stability

- Computational method FireProt

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

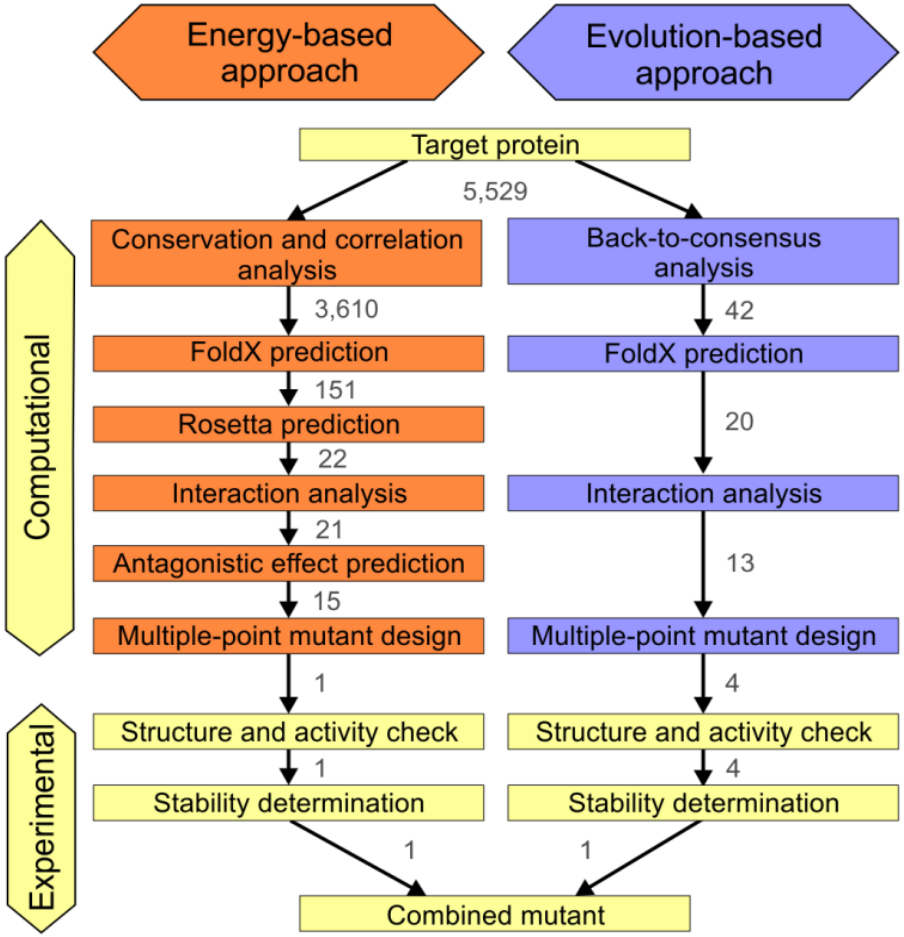
  - prediction of all single-point mutants by FoldX, Rosetta, and back-to-consens
  - smart filtering based on conservation, correlation, electrostatic interactions, and antagonistic effect
  - final prediction of multiple-point mutants for gene synthesis



Design of stability

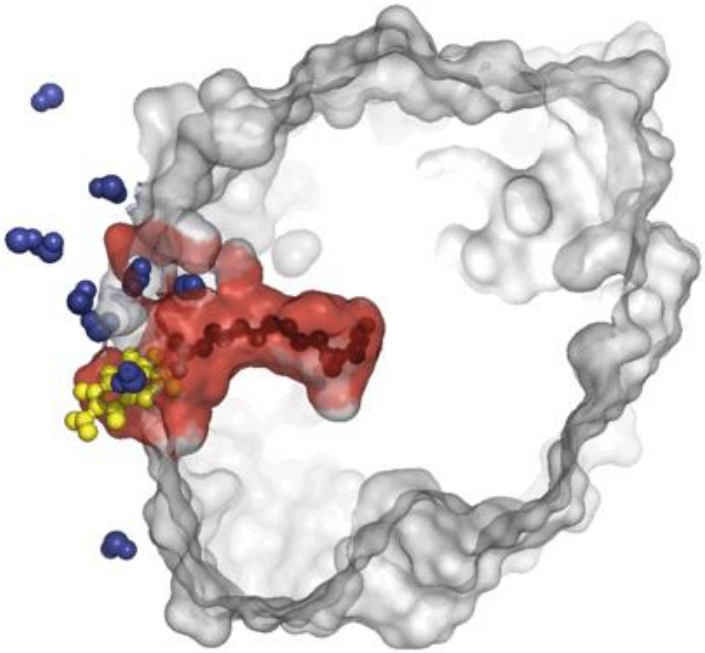
Design of stability – use case

- 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



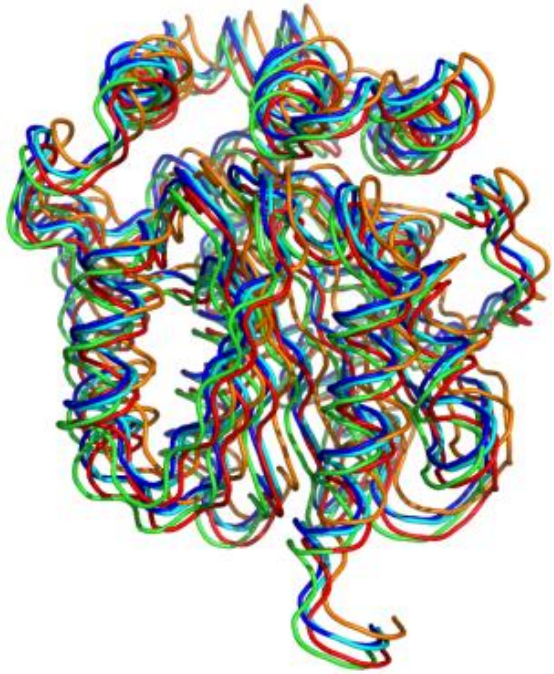
Molecular dynamics

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

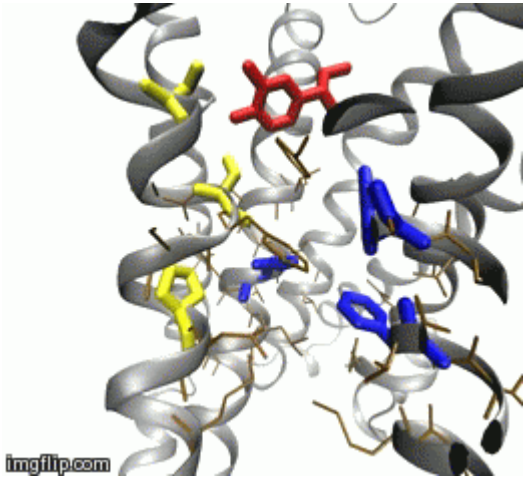


Molecular dynamics

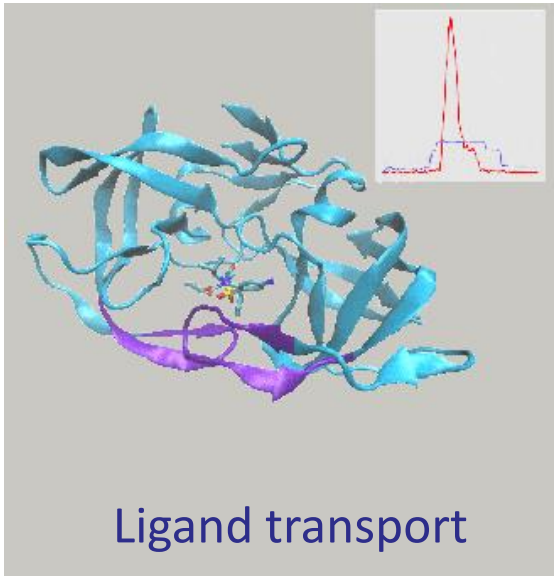
- generates ensemble of structures
  - more precise calculations of free energies



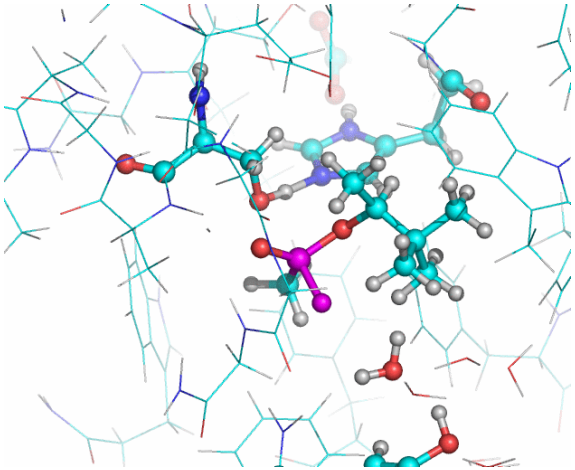
Molecular dynamics



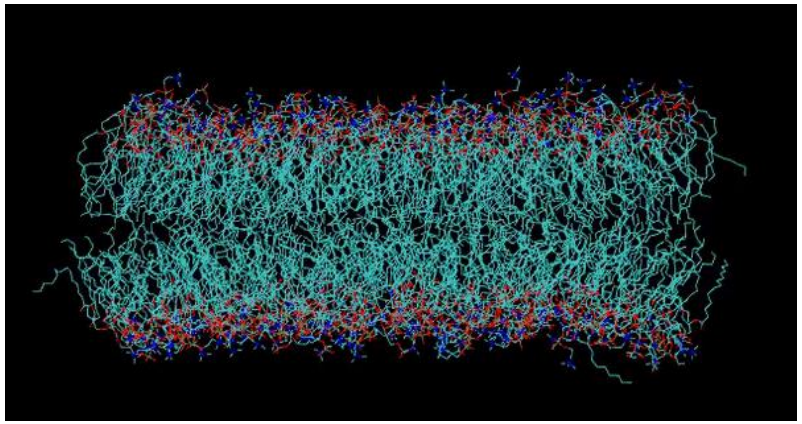
Analysis of interactions



Ligand transport



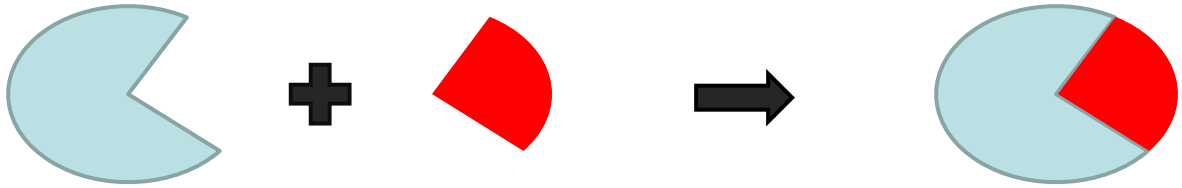
Ligand conversion



Dynamical behaviour

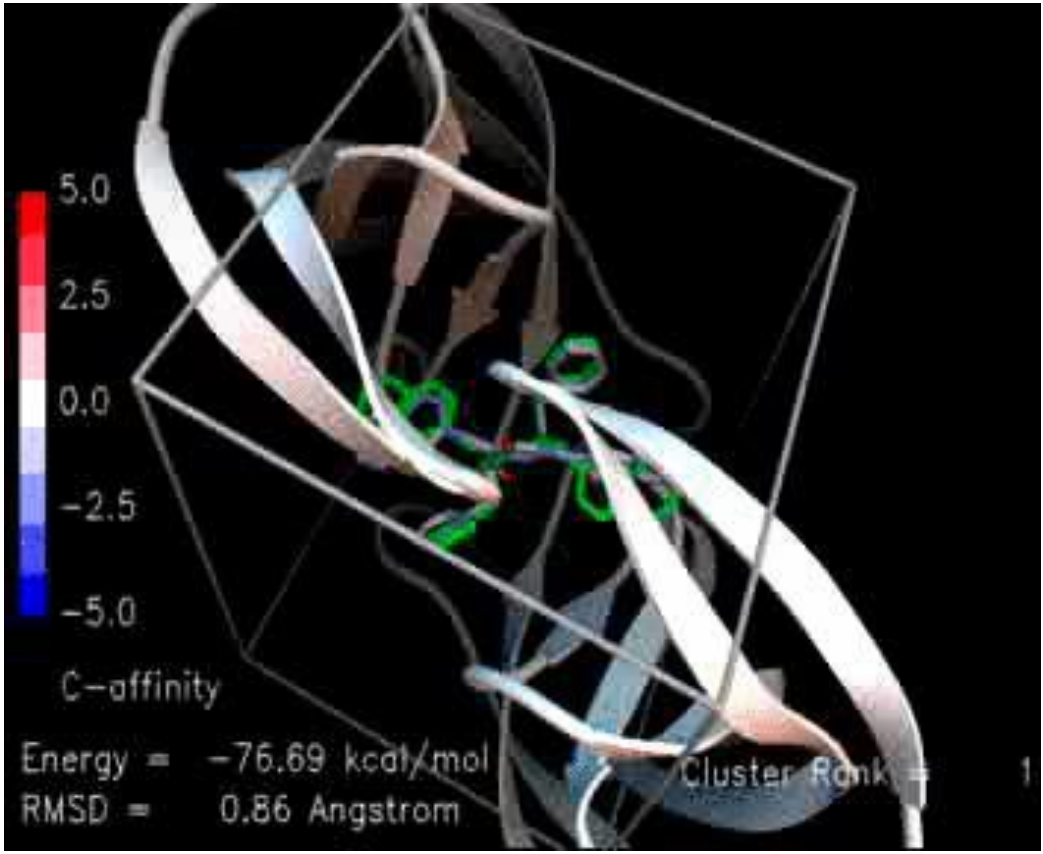
Molecular docking

- predicts structure of receptor (protein) – ligand complex



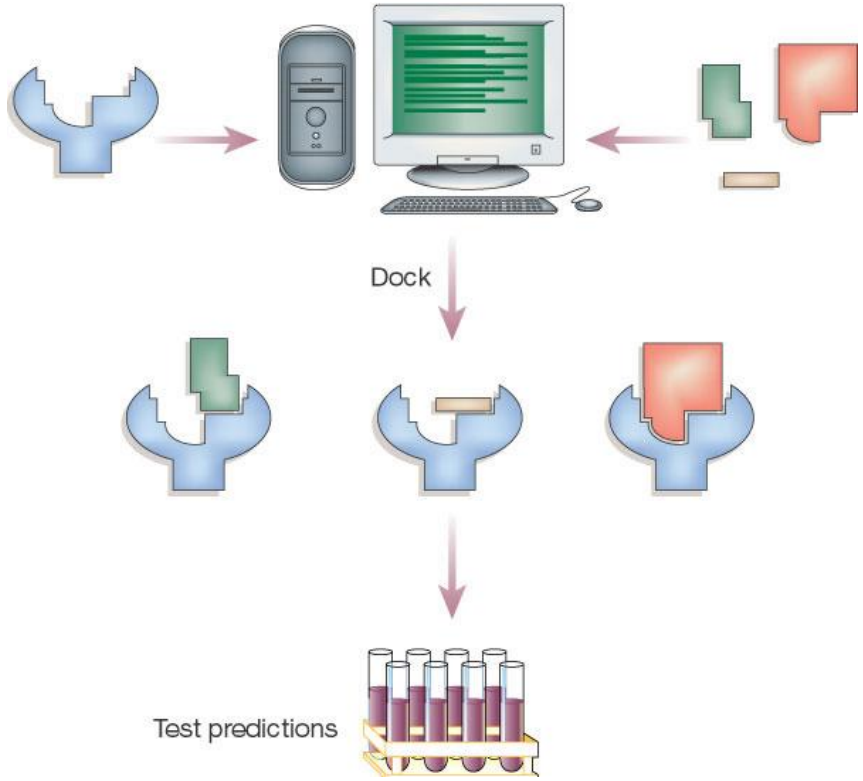
Molecular docking

- 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…



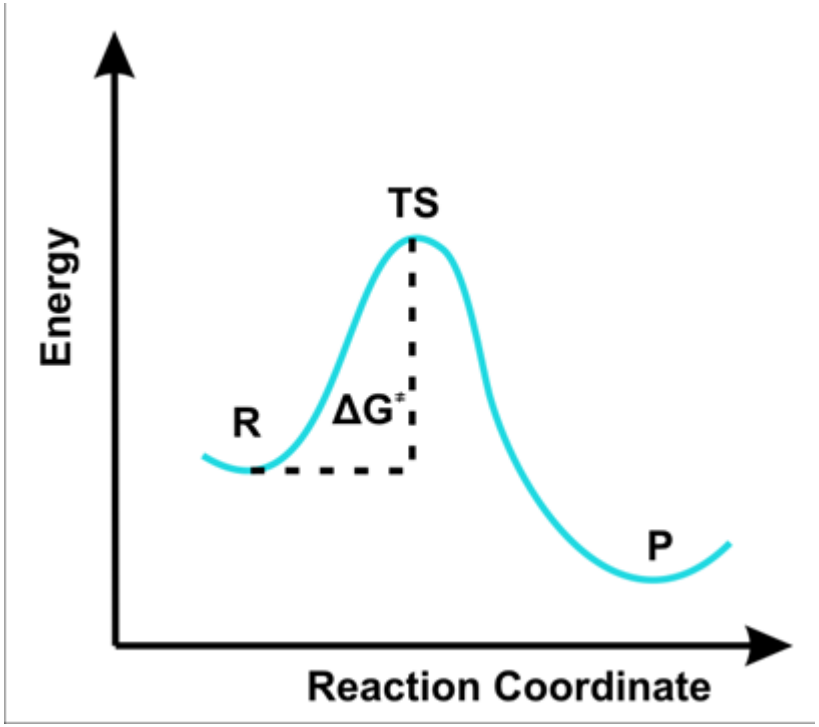
Molecular docking

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



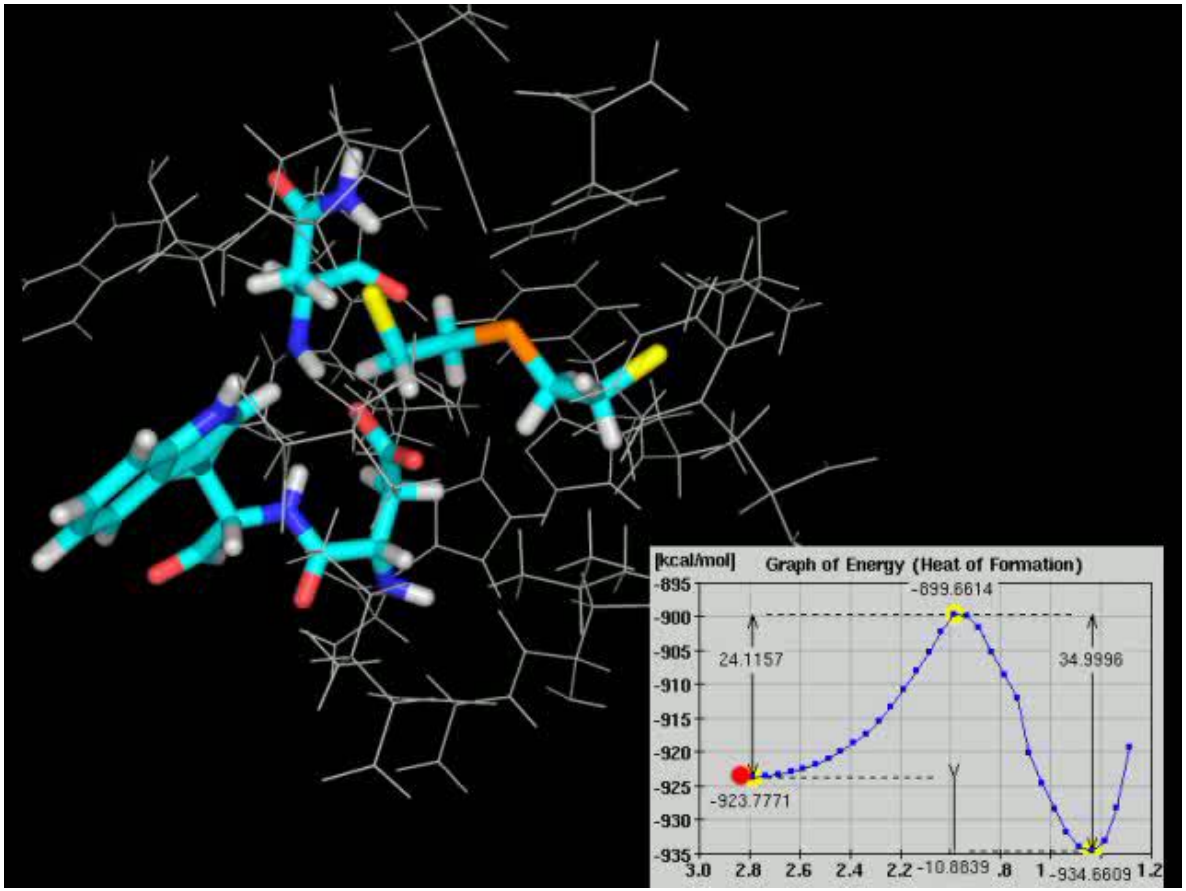
Quantum chemistry

- modeling of reaction
  - reaction barrier



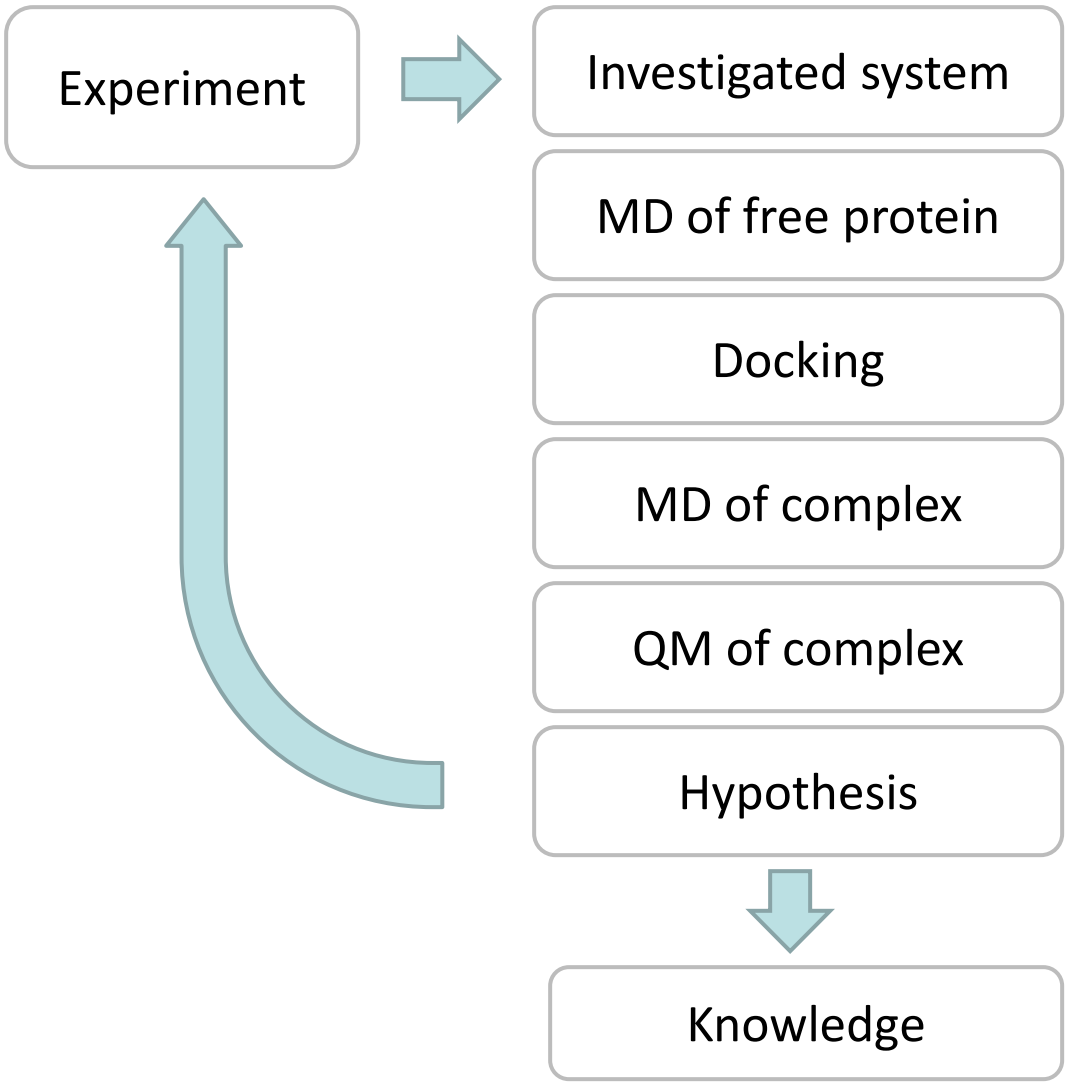
Quantum chemistry

- modeling of reaction



TRITON

Design of mutations

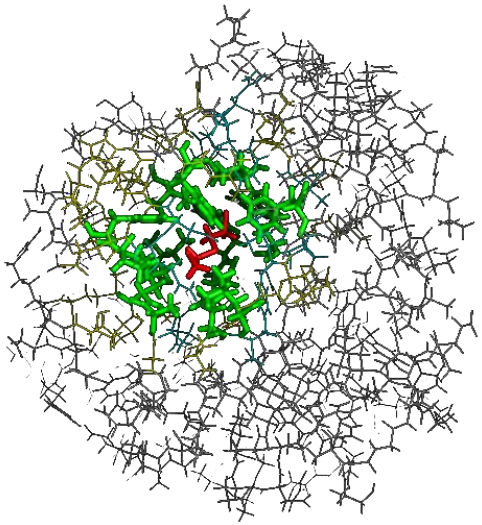


Design of mutations

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

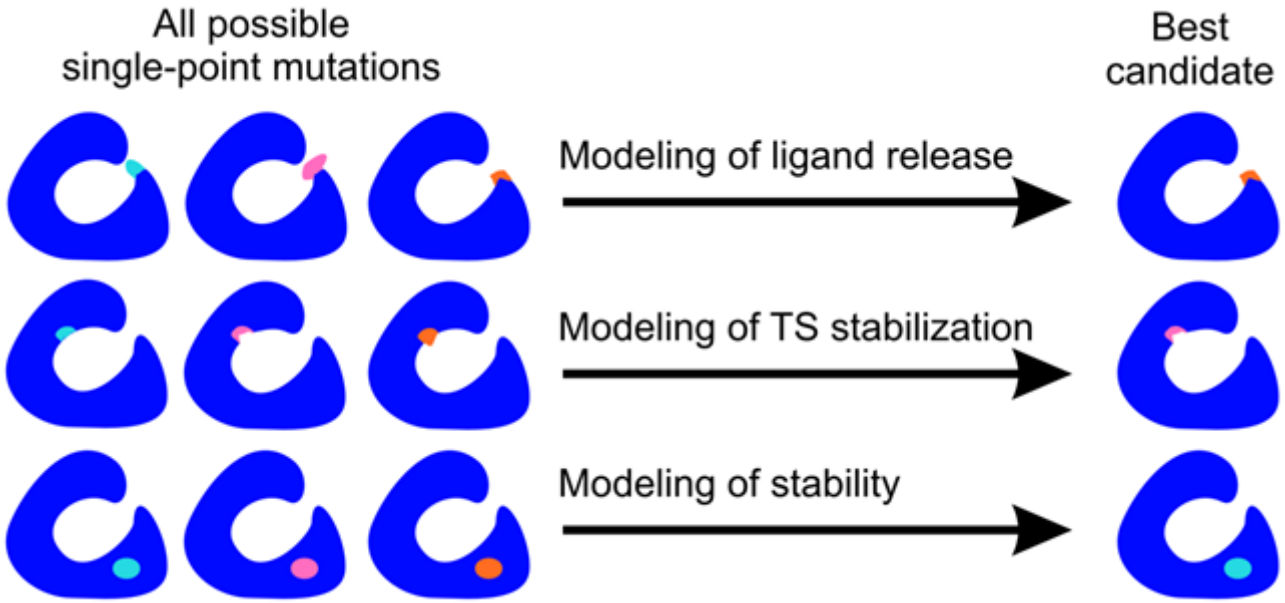
→ further molecular modeling

→ semi-rational design



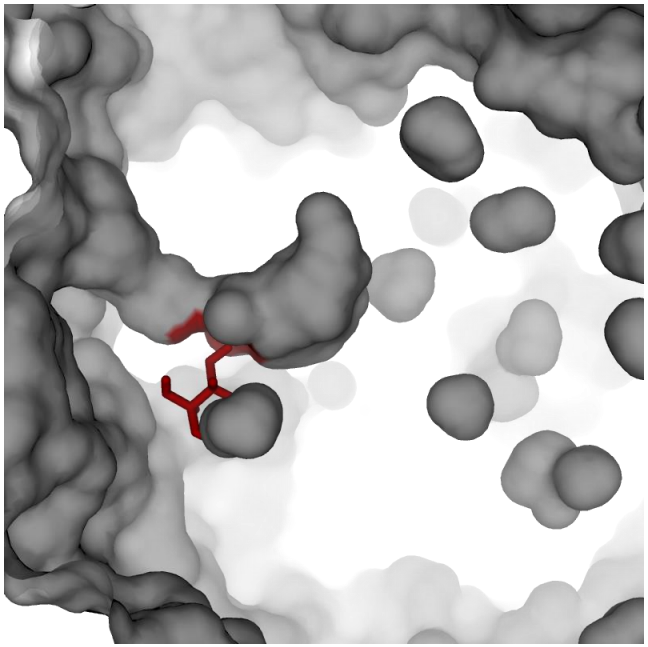
Design of mutations

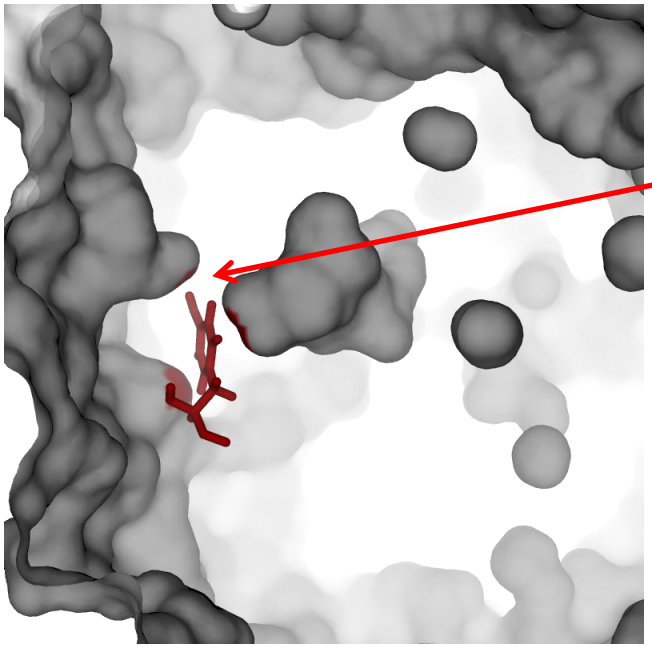
- design of modified enzymes by *in silico* screening
  - study of effects of all relevant mutations
  - selection and combination of the best mutations



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