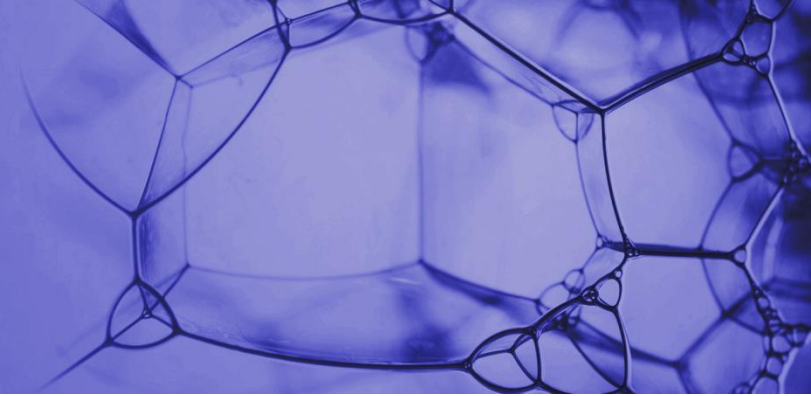


**LOSCHMIDT
LABORATORIES**



Protein Engineering

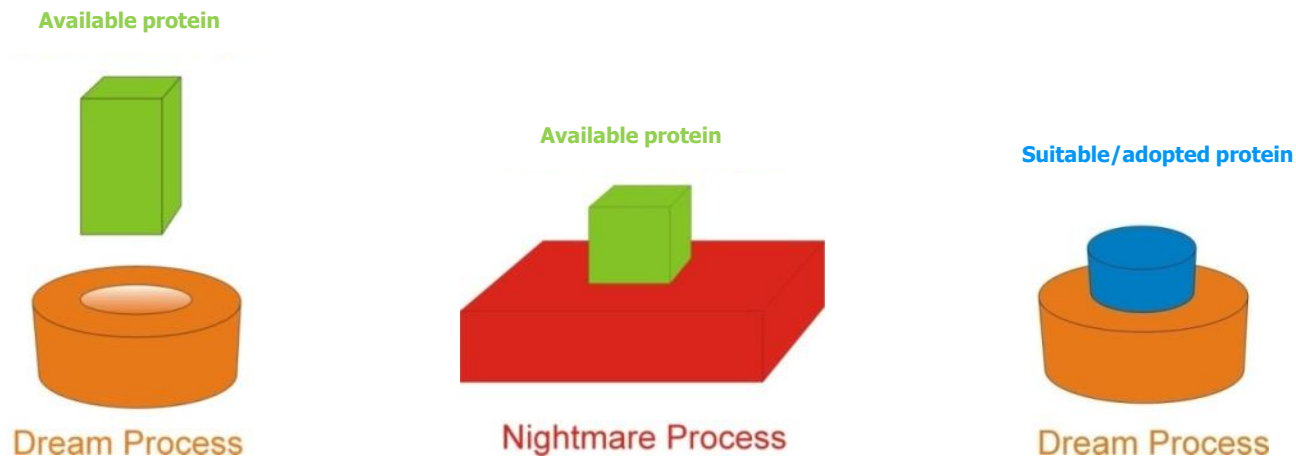
Outline

- ❑ Limitations of proteins in biotechnology processes
- ❑ Definition and aim of protein engineering
- ❑ Targeted properties of proteins
- ❑ Basic approaches in protein engineering
 - **DIRECTED EVOLUTION**
 - **RATIONAL DESIGN**
 - **SEMI-RATIONAL DESIGN**
- ❑ Examples, application of artificial intelligence

Proteins in biotechnology

- ❑ **key problem** -availability of optimal protein for specific process
- ❑ **traditional biotechnology** - adapt process
- ❑ **modern biotechnology** - adapt protein

HOW TO OBTAIN OPTIMAL PROTEIN?



Proteins in biotechnology

classical screening

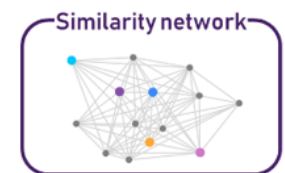
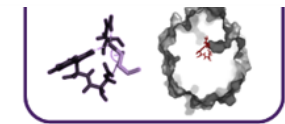
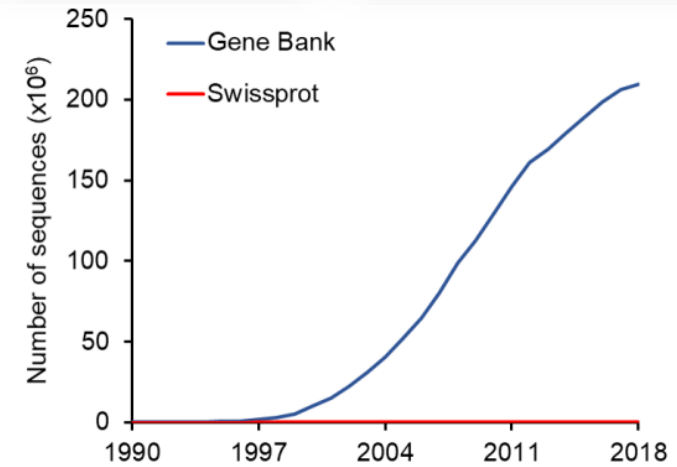
- screening culture collections
- polluted and extreme environment

environmental gene libraries

- metagenomic DNA

data-base mining

- gene databases
- (meta)genome sequencing projects
- numerous uncharacterised proteins



Proteins in biotechnology

- ❑ **classical screening**
 - screening culture collections
 - polluted and extreme environment
- ❑ **environmental gene libraries**
 - metagenomic DNA
- ❑ **data-base mining**
 - gene databases
 - (meta)genome sequencing projects
 - numerous uncharacterised proteins



IF SUITABLE PROTEIN DOES NOT EXIST IN NATURE?

- ❑ **PROTEIN ENGINEERING**

Aims of protein engineering

- ❑ the process of **constructing novel protein** molecules by design first principles or altering existing structure
- ❑ use of genetic manipulations to alter the coding sequence of a gene and thus **modify the properties of the protein**

- ❑ AIMS AND APPLICATIONS
 - **technological** - optimisation of the protein to be suitable in particular technology purpose
 - **scientific** - desire to understand what elements of proteins contribute to folding, stability and function

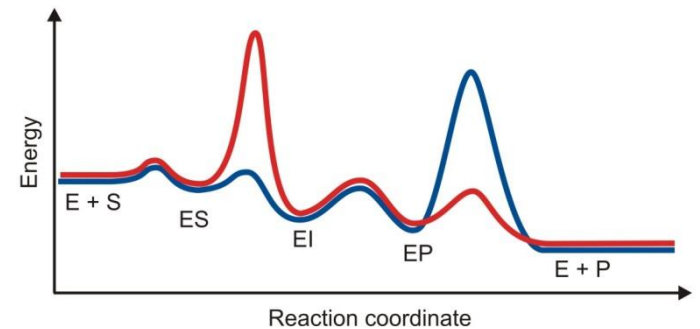
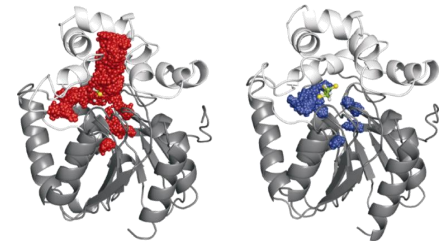
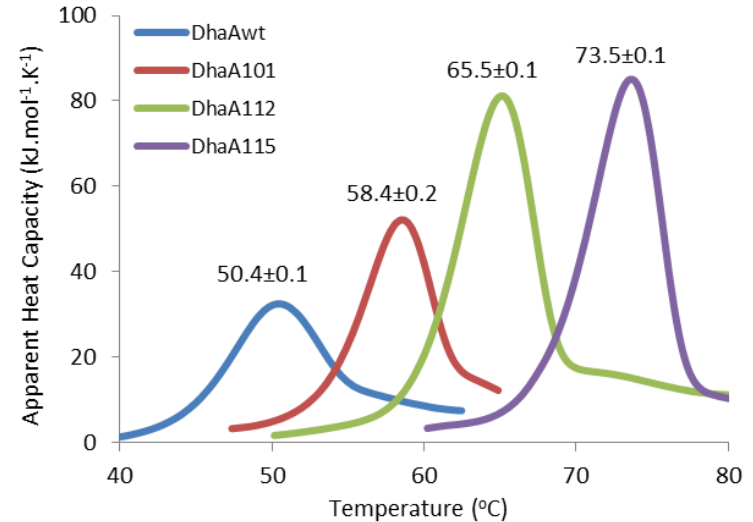
Targeted properties of proteins

□ structural properties of proteins

- **stability** (temperature, solvents)
- tolerance to pH, salt
- resistance to oxidative stress

□ functional properties of proteins

- **substrate specificity** and selectivity
- **kinetic properties** (e.g., K_m , k_{cat} , K_i)
- cofactor selectivity
- protein-protein or protein-DNA interactions



Strategies in protein engineering

RATIONAL DESIGN

1. Computer aided design



2. Site-directed mutagenesis



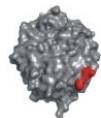
Individual mutated gene

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)

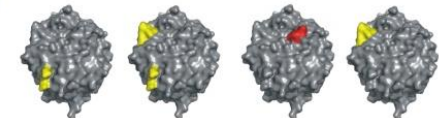
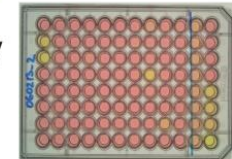
3. Transformation

4. Protein expression

5. *not applied*

6. Screening and selection

- stability
- selectivity
- affinity
- activity



Selected mutant enzymes

Improved
protein

7. Biochemical testing

Directed evolution

- ❑ directed evolution techniques emerged during mid-1990s
- ❑ **inspired by natural evolution**
- ❑ this form of "evolution" does not match what Darwin had envisioned
 - requires **outside intelligence**, not blind chance
 - does not take millions of years, but **happens rapidly**

Frances H. Arnold



Frances H. Arnold
The Nobel Prize in Chemistry 2018

Born: 25 July 1956, Pittsburgh, PA, USA

Affiliation at the time of the award: California Institute of Technology (Caltech), Pasadena, CA, USA

Prize motivation: "for the directed evolution of enzymes."

Prize share: 1/2

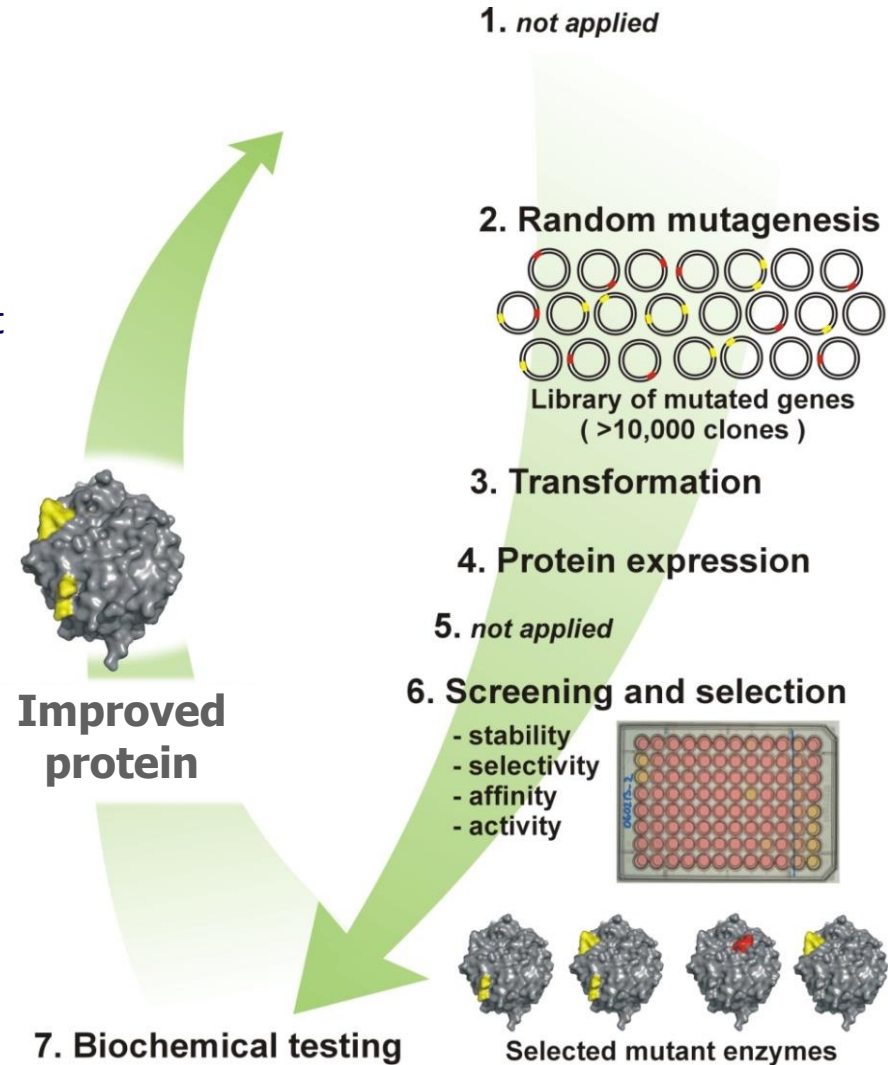
Directed evolution

□ evolution in test tube comprises two steps

- **random mutagenesis**
building mutant library (diversity)
- **screening and selection**
identification of desired biocatalyst

□ prerequisites for directed evolution

- gene encoding protein of interest
- method to create mutant library
- suitable expression system
- screening or selection system

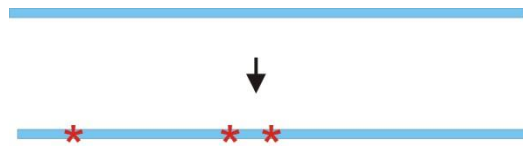


Methods to create mutant libraries

□ technology to **generate large diversity**

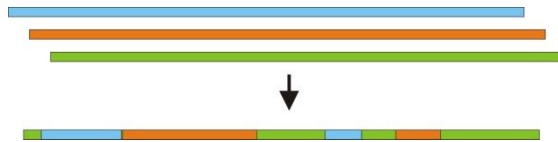
▪ **NON-RECOMBINING**

one parent gene -> variants with point mutations



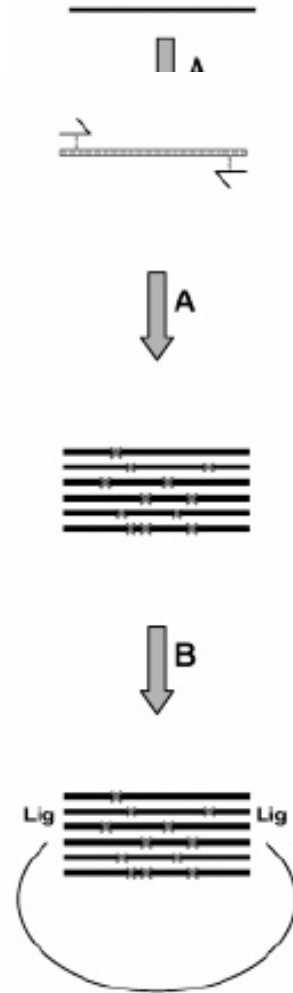
▪ **RECOMBINING**

several parental homologous genes -> chimeras



Non-recombining mutagenesis

- ❑ **UV irradiation or chemical mutagens** (traditional)
- ❑ **mutator strains** - lacks DNA repair mechanism
mutations during replication (e.g., *Epicurian coli* XL1-Red)
- ❑ **error-prone polymerase chain reaction (ep-PCR)**
 - gene amplified in imperfect copying process
(e.g., unbalanced deoxyribonucleotides concentrations,
high Mg^{2+} concentration, Mn^{2+} , low annealing temperatures)
 - 1 to 20 mutation per 1000 base pairs
- ❑ **saturation mutagenesis**
 - randomization of single or multiple codons
 - gene site saturation mutagenesis
- ❑ **other methods**
 - insertion/deletions (InDel)
 - cassette mutagenesis (region mutagenesis)



Recombining mutagenesis

❑ also referred to as „sexual mutagenesis“

❑ **DNA shuffling**

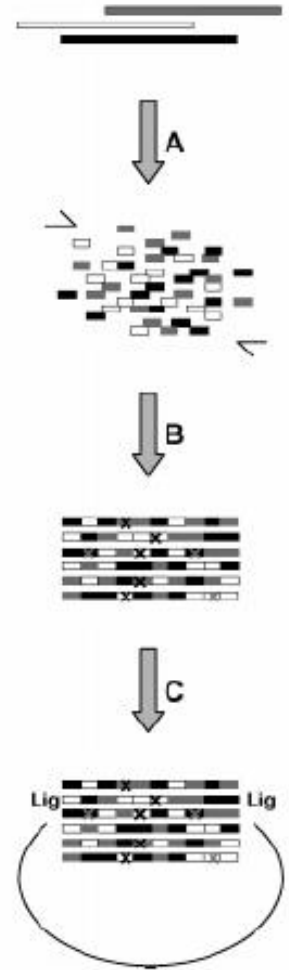
- fragmentation step
- random reassembly of segments

❑ **StEP** - staggered extension process

- simpler than shuffling
- random reannealing combined with limited primer extension

❑ **other methods**

shuffling of genes with lower homology down to 70%
(e.g., RACHITT, ITCHY, SCRATCHY)



Screening and selection

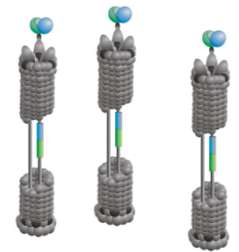
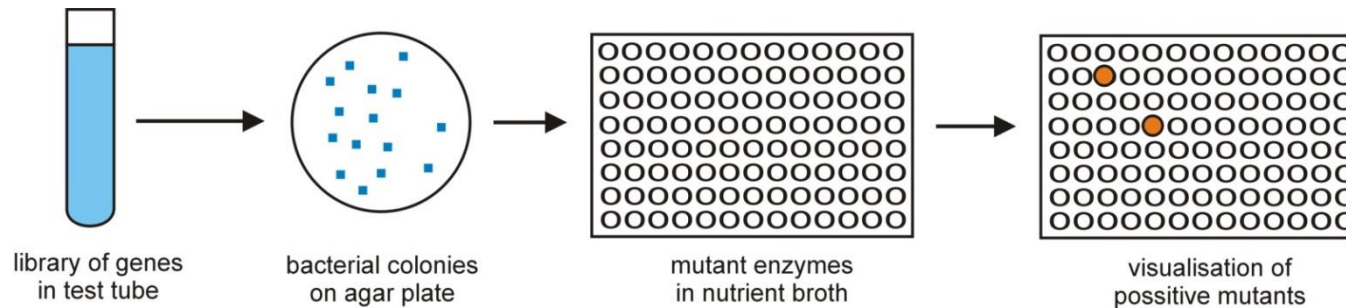
- ❑ most **critical step** of direct evolution
- ❑ isolation of positive mutants hiding in library

- **HIGH THROUGHPUT SCREENING**

individual assays of variants one by one

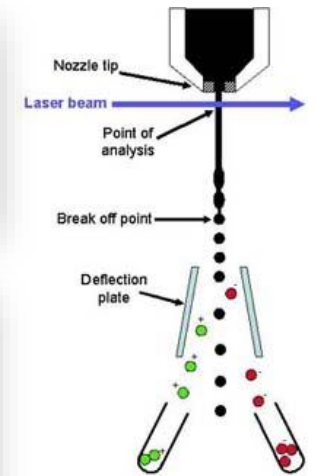
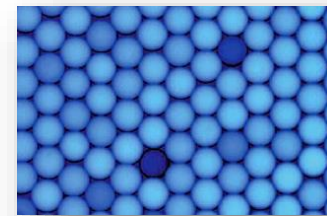
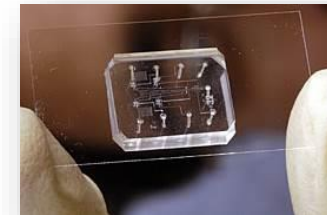
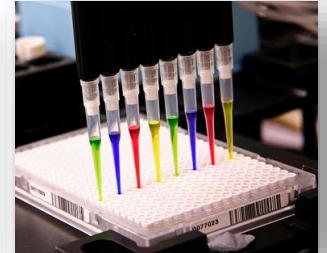
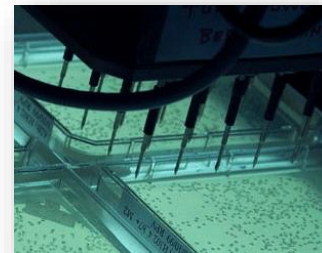
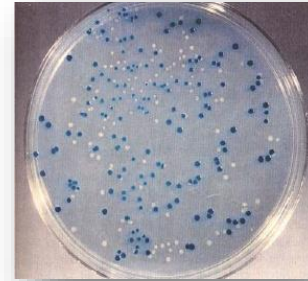
- **DIRECT SELECTION**

display techniques (link between genotype and phenotype)



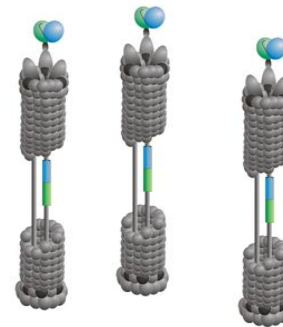
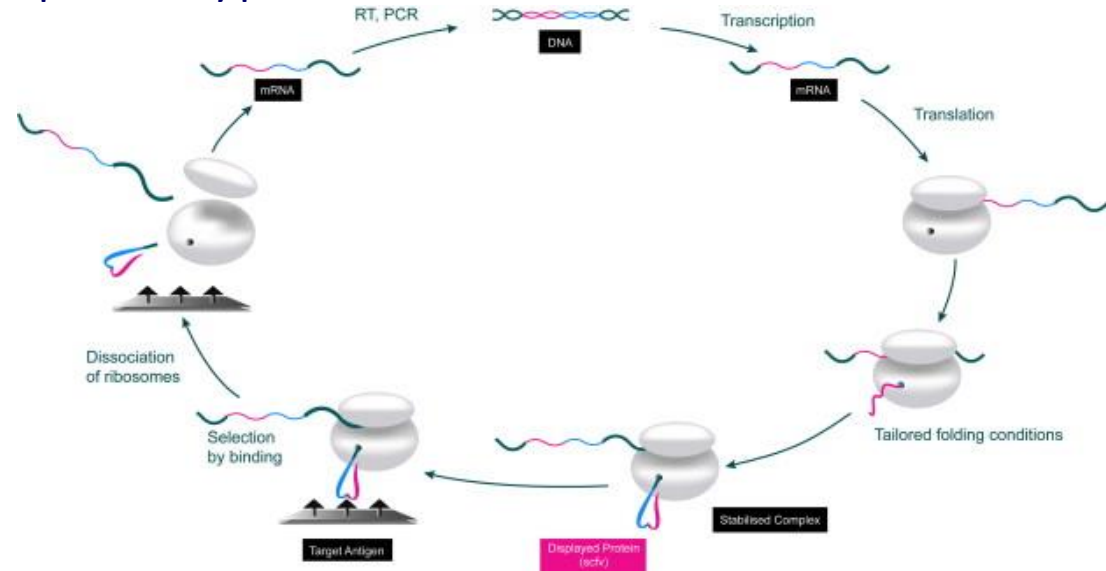
(Ultra)High throughput screening

- ❑ common methods not applicable
- ❑ **agar plate (pre)screening**
- ❑ **microtiter plates screening**
 - 96-, 384- or 1536-well formate
 - robot assistance
(colony picker, liquid handler)
 - 10^4 libraries
 - volume 10 – 100 μ L
- ❑ **microfluidic systems (*Lesson 6*)**
 - water in oil emulsions (up to 10 kHz)
 - FACS sorting (10^8 events/hour)
 - 10^9 libraries
 - volume 1 – 10 pL



Direct selection

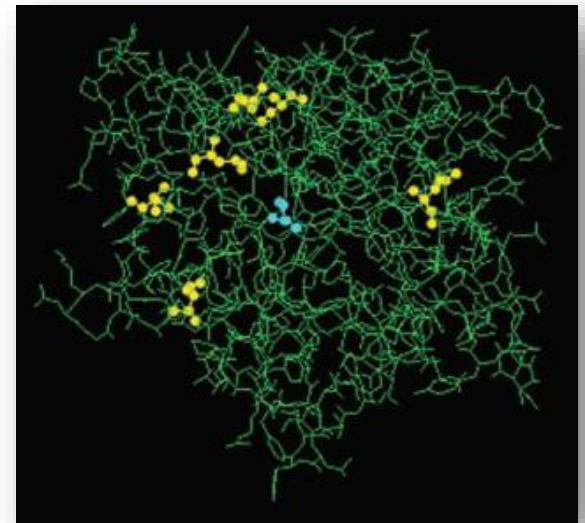
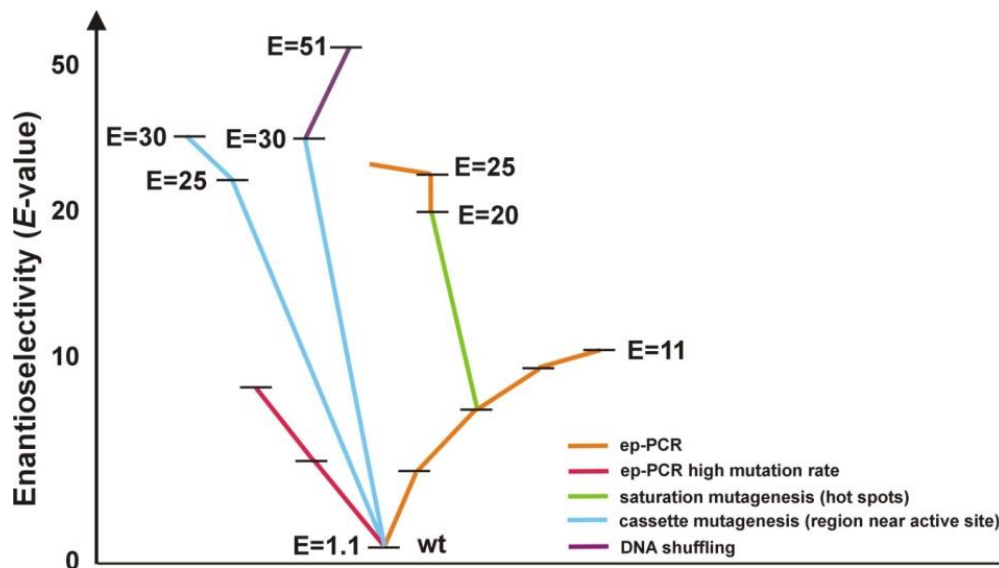
- ❑ not generally applicable (mutant libraries $>10^6$ variants)
- ❑ link between genotype and phenotype
- ❑ **display technologies**
 - ribosome display
 - phage display
- ❑ **life-or-death assay**
 - auxotrophic strain
 - toxicity based selection



Example of Directed evolution

□ directed evolution of **enantioselectivity**

- lipase from *P. aeruginosa* (E-value improved from 1.1 into 51)
- **spectrophotometric screening** of (*R*)- and (*S*)-nitrophenyl esters
- **40 000 variants** screened
- the best mutant contains six amino acid substitutions



Strategies in protein engineering

RATIONAL DESIGN

1. Computer aided design



2. Site-directed mutagenesis



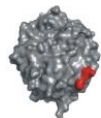
Individual mutated gene

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)

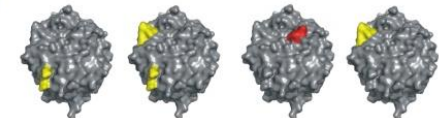
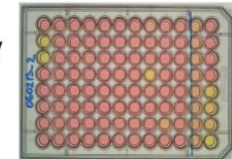
3. Transformation

4. Protein expression

5. *not applied*

6. Screening and selection

- stability
- selectivity
- affinity
- activity



Selected mutant enzymes

Improved
protein

7. Biochemical testing

Rational design

- ❑ emerged around 1980s as the original protein engineering approach
- ❑ **knowledge based** - combining theory and experiment
- ❑ protein engineering cycle:
„structure-theory-design-mutation-purification-analysis“
- ❑ **difficulty in prediction** of mutation effects on protein property
- ❑ **de novo design** most challenging

Principal of rational design

1. Computer aided design



2. Site-directed mutagenesis



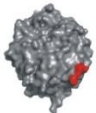
Individual mutated gene

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4. Protein expression

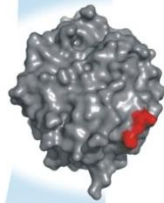
5. Protein purification

6. *not applied*



Constructed mutant enzyme

7. Biochemical testing



Improved protein

□ rational design comprises:

- **design** - understanding of protein functionality
- **experiment** - construction and testing of mutants

□ prerequisites for rational design:

- **gene** encoding protein of interest
- **3D structure** (e.g., X-ray, NMR) or sequence alignment
- structure-function relationship
- computational methods and capacity
- side directed mutagenesis techniques
- efficient expression system
- biochemical tests

Design

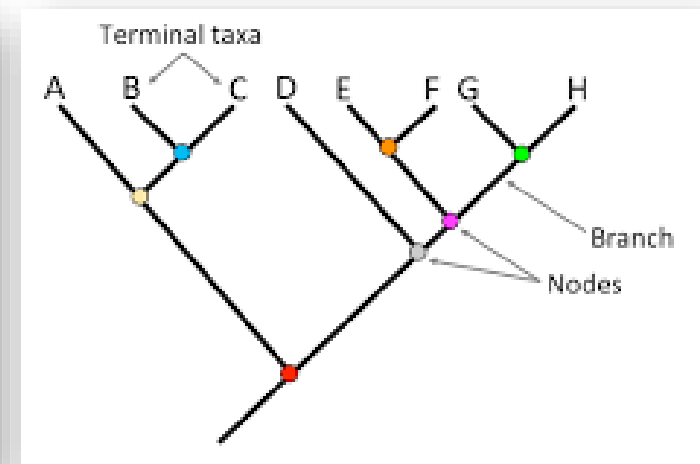
□ SEQUENCE HOMOLOGY APPROACH

- homologous wild-type **sequences alignment**
- **identifying** amino acid residues responsible for differences
- **design** - combination of positive mutation from all parental proteins

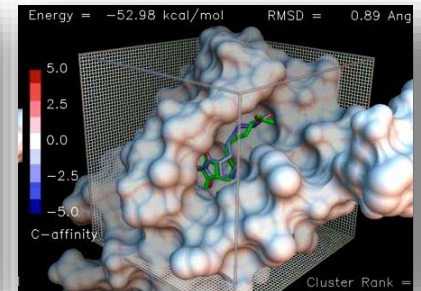
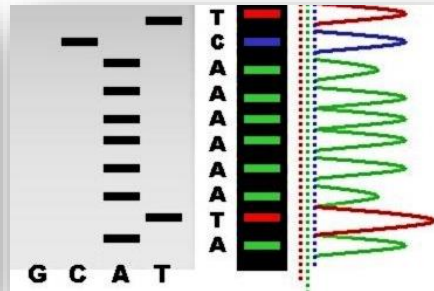
□ ANCESTRAL RECONSTRUCTION

- construction of **phylogenetic tree**
- **design** - nodes prediction by **consensus approach**

RLA0 DICDI	-----HSCAG-SKRKLFIEKATKLFITTDKMIVAEADFYGSSLOLQIRKSI	INGI-GAVLMGKKMIRKVI	INDLADSK--PELD	75		
Q54LPO DICDI	-----HSCAG-SKRKNVFEKATKLFITTDKMIVAEADFYGSSLOLQIRKSI	INGI-GAVLMGKKMIRKVI	INDLADSK--PELD	75		
RLA0 PLAFB	-----MAKLSKQKKQMYIEKLSLQQQSKILIVHVDNVCNEMASVYKSLGK	-AETLMGKTRIRITALKK	NAV-POI	76		
RLA0 SULAC	-----MIGLAVTTTKKIAPKQVDEVAELTSLKTRTKTIIIANIEGFPADKL	HEIRKKL	LRGK-ADIKVTKNL	IFNIALKNAG--VD	79	
RLA0 SULTO	-----HRIMAVITQERKIAKWKIEEVEKLEKLRVHTIIIANIEGFPADKL	HDIRK	MRGM-AEIKVTKHL	LPGLAAKNAG--LDVS	80	
RLA0 SULSO	-----MKRLALALKQRKVASWKEEVEKLTSLIKNSNTILINIEGFPADKL	HEIRKKL	LRGK-AEIKVTKHL	LPGLAAKNAG--IDIS	80	
RLA0 AERPE	-----MSVYLVGQMYKREKFLPEKNTLMRELESLFSKRRVFLADLTGSL	FFVDRVYK	KKWKK-VMMVAKKR	IIILAMKAAGLE--LDDN	86	
RLA0 PYRAE	-----HMLAIVGQMYKREKFLPEKNTLMRELESLFSKRRVFLADLTGSL	FFVDRVYK	KKWKK-VMMVAKKR	IIILAMKAAGLE--IPAS	85	
RLA0 METAC	-----MAEERHNTHEIPQWKKDEIENIKELIQSHKVFQMVSI	EGILATKMKIRRD	LKDV-AVLKVS	RNTLLEBALNQLG--ETIP	78	
RLA0 METMA	-----MAEERHNTHEIPQWKKDEIENIKELIQSHKVFQMVSI	EGILATKMKIRRD	LKDV-AVLKVS	RNTLLEBALNQLG--ESIP	78	
RLA0 ARCFU	-----MAAVRES--PDEKVRRAVEIEKRMISSEKVVYAIYDFRNY	PAGDOKIRRE	FRGK-AEIKVT	KHLLEBALD	ALG--GQYL	75
RLA0 METKA	-----HAYKAKSPPSSEYEPKVAEKKRREVEKELLMDEYEMVGLDLE	IPAPDQLEIRAKLR	ERDIIIRMS	RNTLLEBALNQLG--PELD	80	
RLA0 METTH	-----MAHVAEWKKKEVQELNDLKKYEVVYIAHLADIPARQL	OKMOTLRDS-ALIRMS	RNTLLEBALNQLG--EHVD	74		
RLA0 METIL	-----MITAESEHKIADPKIEEYVWKLKELKKNQDIYALVDMMEY	PARDQLEIRDKTR-ETWELKMS	RNTLLEBALNQLG--PEFA	82		
RLA0 METVA	-----MIDAKSEHKIADPKIEEYVWKLKELKKNQDIYALVDMMEY	PARDQLEIRDKTR-DQWELKMS	RNTLLEBALNQLG--PEFA	82		
RLA0 METJA	-----METYVKAHVADPKIEEYVWKLKELKKNQDIYALVDMMEY	PARDQLEIRDKTR-DKVKLRMS	RNTLLEBALNQLG--PEFA	81		
RLA0 PYRAB	-----MAHVAEWKKKEVEELAKLIKSPYIALVDVSSMPAYPLSQM	RRLIRENNG	LLRVS	RNTLLEBALNQLG--LQKPEL	77	
RLA0 PYRHO	-----MAHVAEWKKKEVEELAKLIKSPYIALVDVSSMPAYPLSQM	RRLIRENNG	LLRVS	RNTLLEBALNQLG--LQKPEL	77	
RLA0 PYRFU	-----MAHVAEWKKKEVEELAKLIKSPYIALVDVSSMPAYPLSQM	RRLIRENNG	LLRVS	RNTLLEBALNQLG--LQKPEL	77	
RLA0 PYRKO	-----MAHVAEWKKKEVEELAKLIKSPYIALVDVSSMPAYPLSQM	RRLIRENNG	LLRVS	RNTLLEBALNQLG--LQKPEL	76	
RLA0 HALMA	-----MSAESEKRTETPEKQEEVDALVDFIESYSEVGVVNIAGIP	RRLODMRR	RLHGT-AELRVS	RNTLLEBALNQLG--DGLS	79	
RLA0 HALVO	-----MSESEVQTEVEIPQWKREVEVDALVDFIESYSEVGVVNIAGIP	RRLODMRR	RLHGT-AAVRRMS	RNTLLEBALNQLG--DGFV	79	
RLA0 HALSA	-----MSAESEKRTETPEKQEEVDALVDFIESYSEVGVVNIAGIP	RRLODMRR	RLHGT-AALRMS	RNTLLEBALNQLG--DGLD	79	
RLA0 THEAC	-----MKEVSQKKEVLENEITLQIKASSVAIVDLAGISRRID	IRGK	NRGK-INLKV	IKKLLF	KALENLGD--EKLS	72
RLA0 THEVO	-----MRRINPKKKEIVSELAALITKSKAVAIVDIKGVSRRID	IRGK	NRGK-VKIKV	IKKLLF	KALDSND--EKLT	72
RLA0 PICTO	-----MTEPAQMKIDFYKNLENIENSRKVAIVSIKLIRNHF	DKIRNS	IRDK-ARIK	VSRARLL	LALENLQK--NHIV	72
ruler	1.....10.....20.....30.....40.....50.....60.....70.....80.....90					

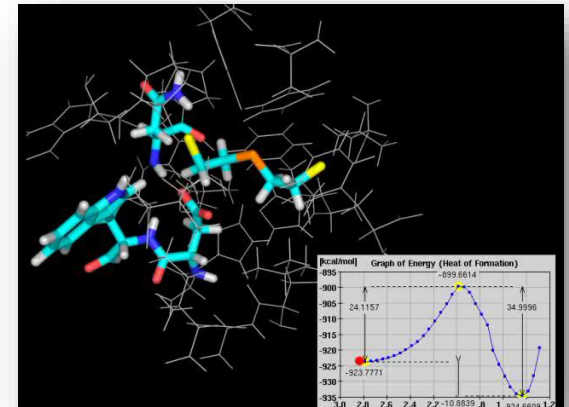
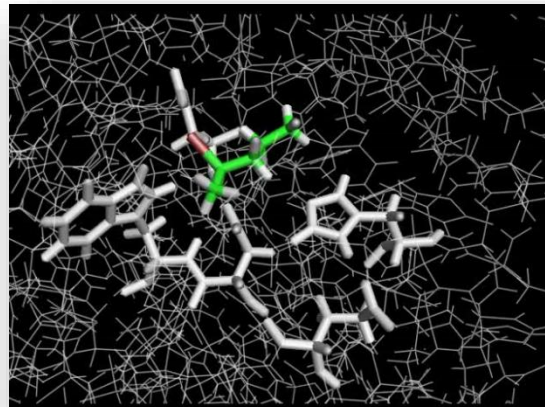
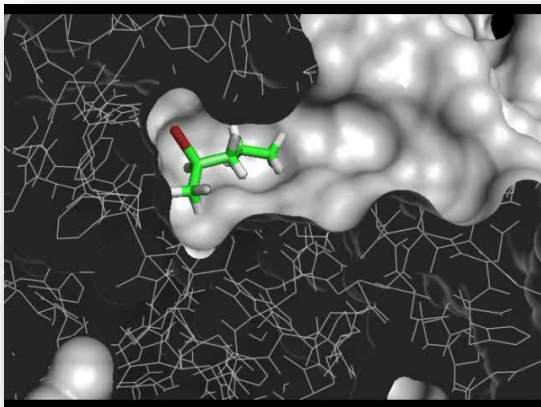


- Období: podzim
- Rozsah: přednáška 2 hodiny/týden, cvičení 2 hodiny/týden
- Vyučující: prof. Mgr. Jiří Damborský, Dr., doc. RNDr. Roman Pantůček, Ph.D.,
- Osnova:
 - bioinformatické databáze a jejich prohledávání
 - analýza nukleotidových a proteinových sekvencí
 - hledání a identifikace genů
 - analýza a předpověď struktury proteinů



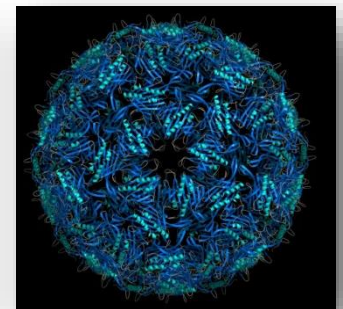
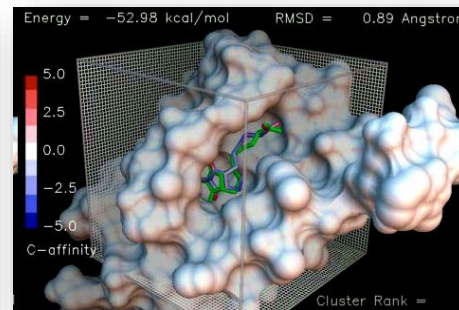
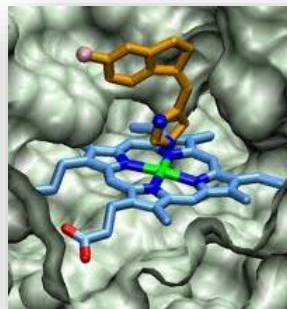
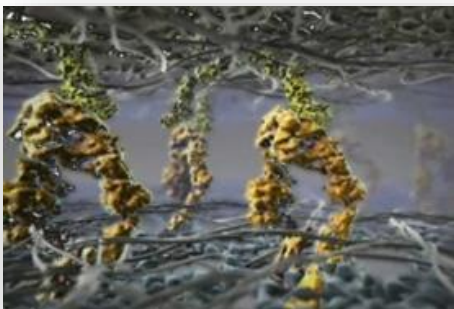
❑ STRUCTURE-BASED APPROACH

- **prediction** of enzyme function from structure alone is challenging
- **protein structure** (X-ray crystallography, NMR, *homology models!*)
- **molecular modelling**
 - molecular docking
 - molecular dynamics
 - quantum mechanics/molecular mechanics (QM/MM)



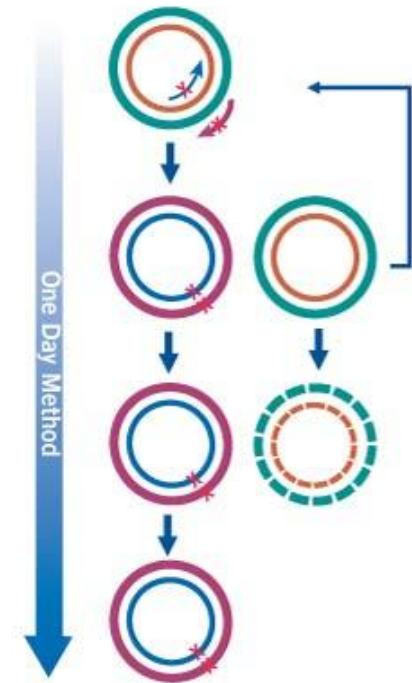
Strukturní biologie Bi9410

- Období: podzim
- Rozsah: přednáška 2 hodiny/týden, cvičení 2 hodiny/týden
- Vyučující: Mgr. David Bednář
- Osnova:
 - struktura, stabilita a dynamika biologických makromolekul
 - makromolekulární interakce a komplexy
 - stanovení a předpověď struktury, identifikace důležitých oblastí
 - stanovení vlivu mutace na strukturu a funkci proteinu
 - aplikace v biologickém výzkumu, návrhu léčiv a biokatalyzátorů



Construction

- ❑ **site-directed mutagenesis**
 - introducing point mutations
- ❑ **multi site-directed mutagenesis**
- ❑ **gene synthesis**
 - commercial service
 - codone optimisation



GENEART
THE GENE OF YOUR CHOICE

GenScript
Make Research Easy

Example of rational design

□ rational design of protein **stability**

- stability to high temperature, extreme pH, proteases etc.
- **stabilizing mutations** increase strength of weak interactions

- **salt bridges and H-bonds**

Eijsink et al., Biochem. J. 285: 625-628, 1992

- **S-S bonds**

Matsumura et al., Nature 342: 291-293, 1989

- **addition of prolines**

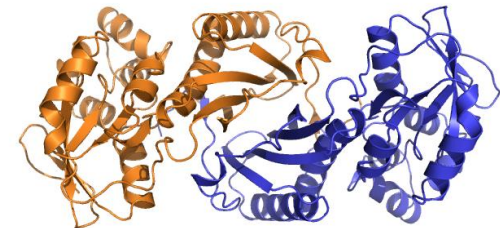
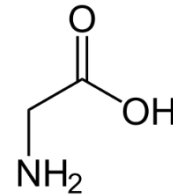
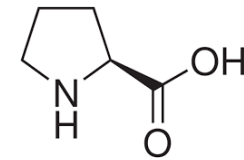
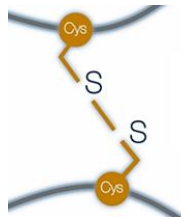
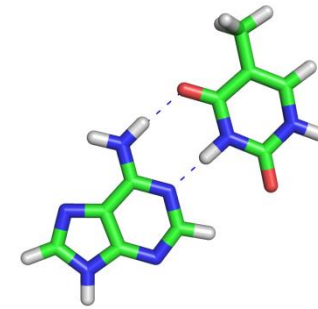
Watanabe et al., Eur. J. Biochem. 226: 277-283, 1994

- **less glycines**

Margarit et al., Protein Eng. 5: 543-550, 1992

- **oligomerisation**

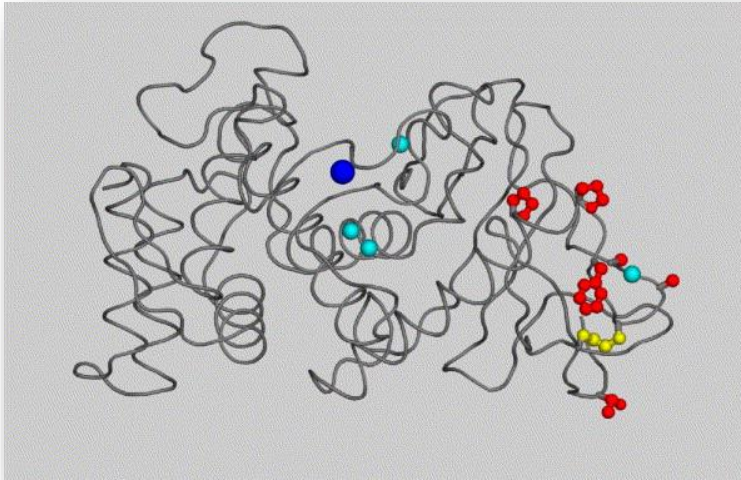
Dalhus et al., J. Mol. Biol. 318: 707-721, 2002



Example of rational design

□ engineering protein to resist boiling

- **reduced rotational freedom**
Ser65Pro, Ala96Pro
- **introduction of disulfide bridge**
Gly8Cys + Asn60Cys
- **improved internal hydrogen bond**
Ala4Thr
- **filling cavity**
Tyr63Phe



Half-lives (min.)	80°C	100°C
wild type	17.5	>0.5
mutant	stable	170

Strategies in protein engineering

RATIONAL DESIGN

1. Computer aided design



2. Site-directed mutagenesis



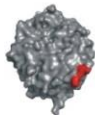
Individual mutated gene

3. Transformation

4. Protein expression

5. Protein purification

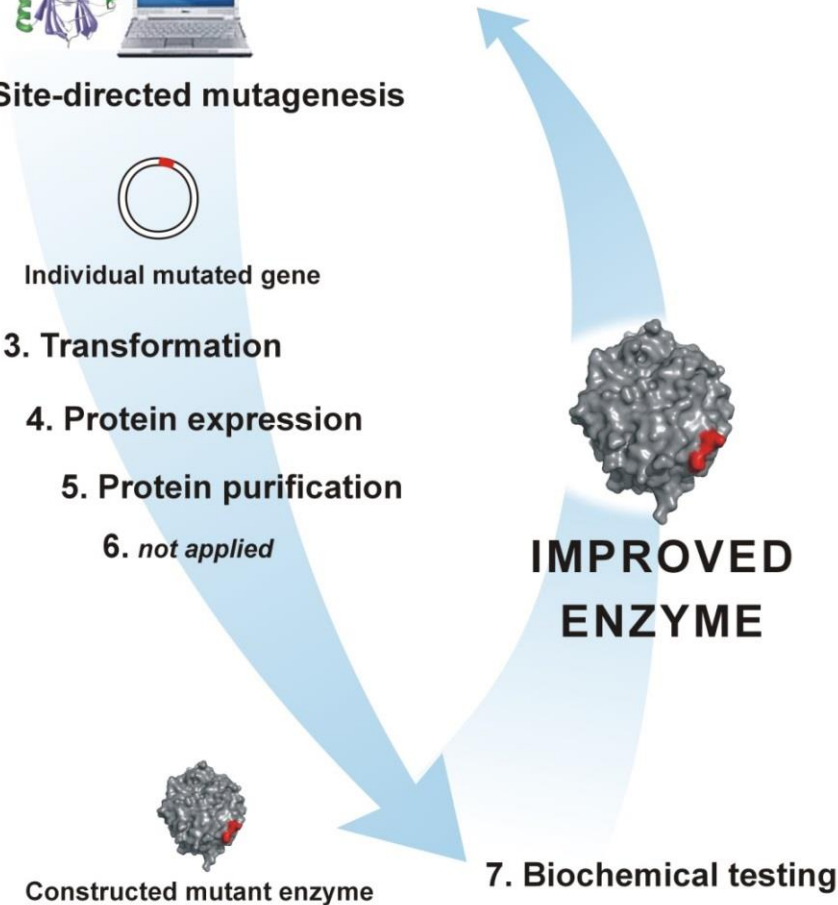
6. *not applied*



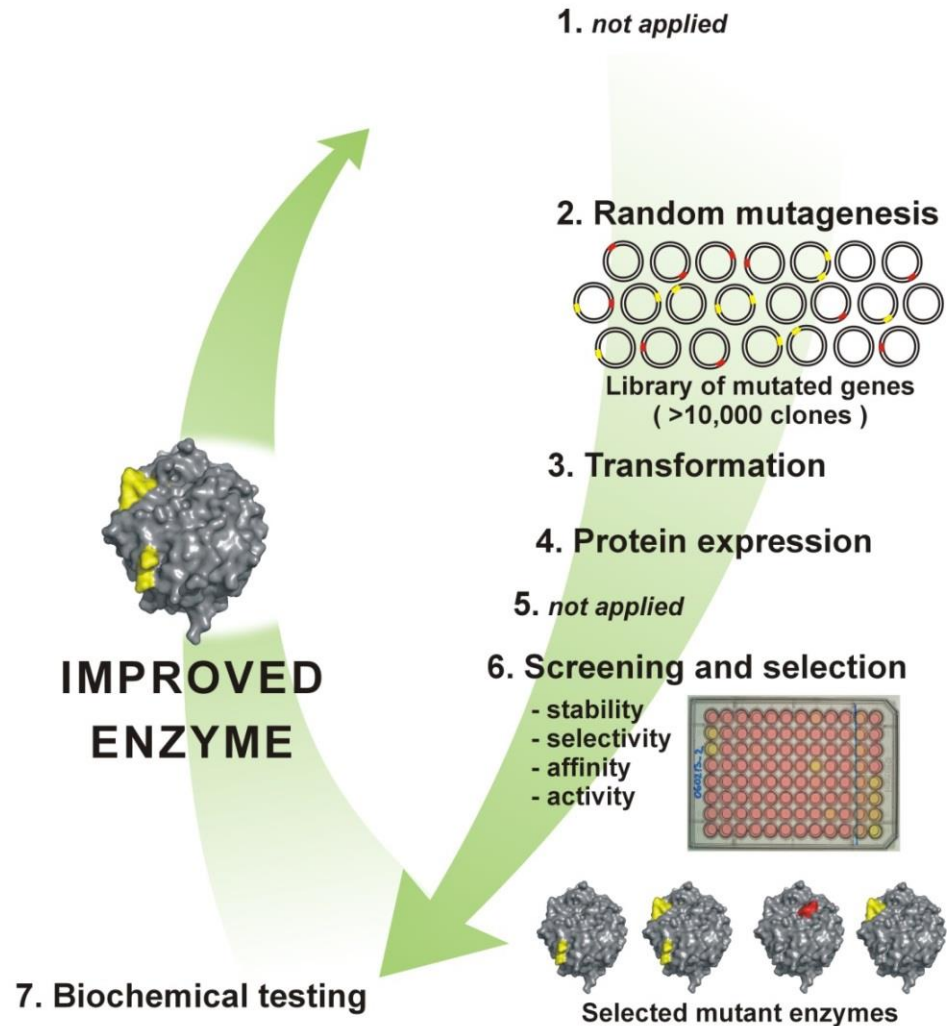
Constructed mutant enzyme

**IMPROVED
ENZYME**

7. Biochemical testing



DIRECTED EVOLUTION



Strategies in protein engineering

RATIONAL DESIGN

1. Computer aided design



2. Site-directed mutagenesis



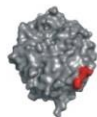
Individual mutated gene

3. Transformation

4. Protein expression

5. Protein purification

6. *not applied*



Constructed mutant enzyme

DIRECTED EVOLUTION

SEMIRATIONAL DESIGN

2. Random mutagenesis



Library of mutated genes
(>10,000 clones)

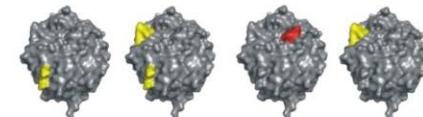
3. Transformation

4. Protein expression

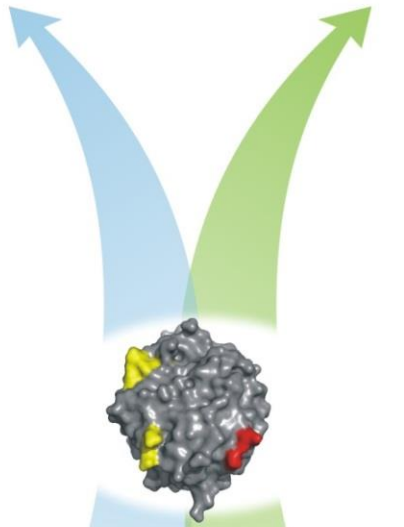
5. *not applied*

6. Screening and selection

- stability
- selectivity
- affinity
- activity



Selected mutant enzymes

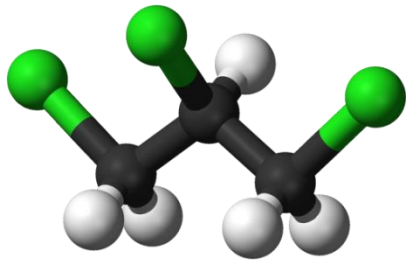


IMPROVED ENZYME

7. Biochemical testing

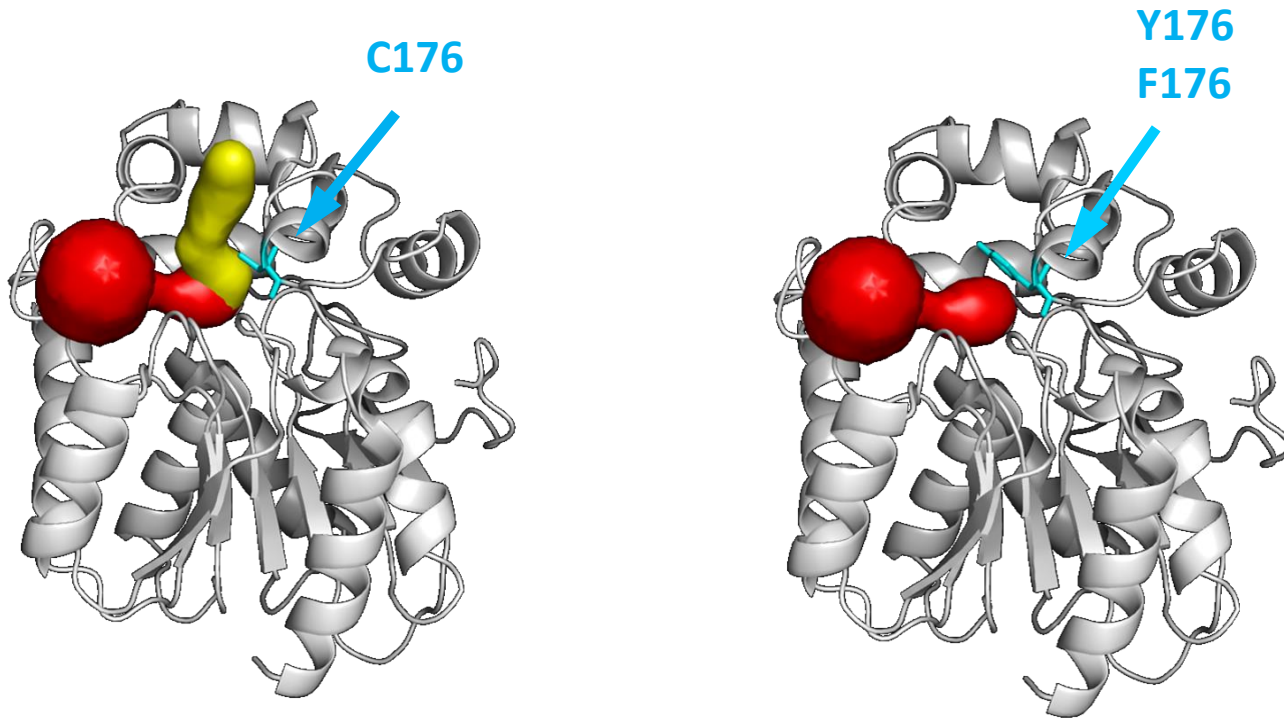
Example of semi-rational design

- conversion of 1,2,3-trichloropropane
by DhaA from *Rhodococcus erythropolis* Y2



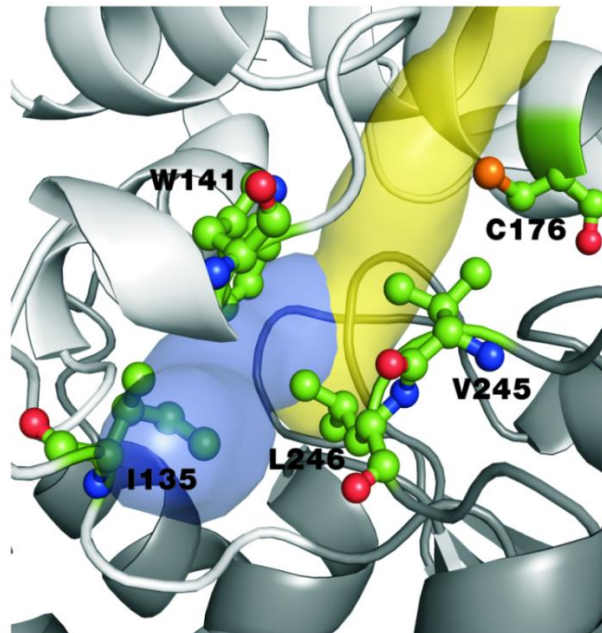
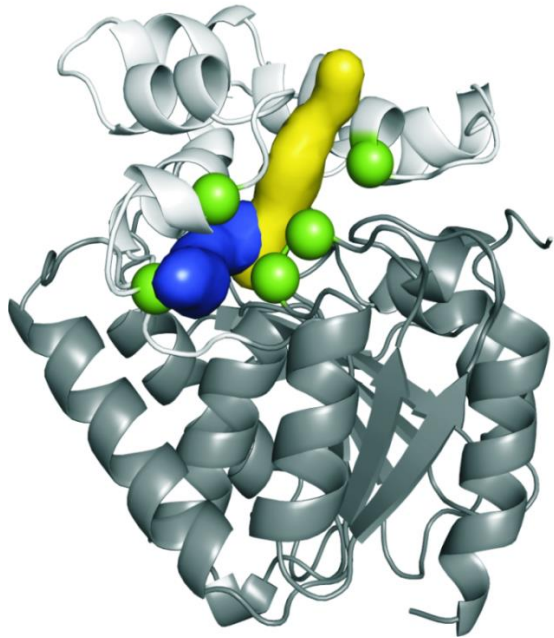
Example of semi-rational design

- ❑ conversion of 1,2,3-trichloropropane by DhaA from *Rhodococcus erythropolis* Y2
- ❑ **DIRECTED EVOLUTION** - importance of access pathways

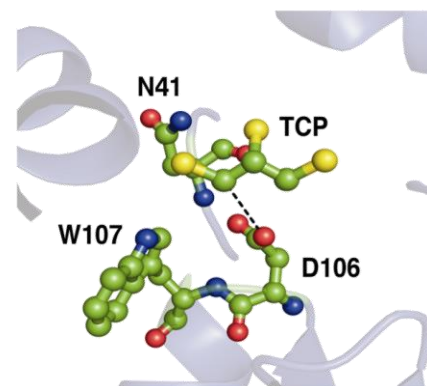
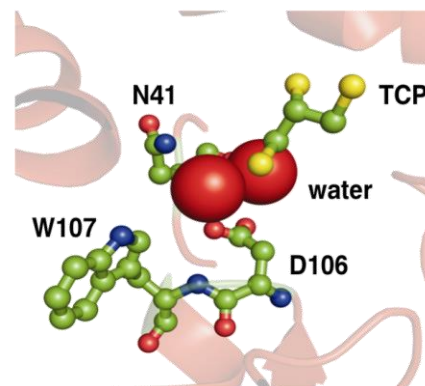
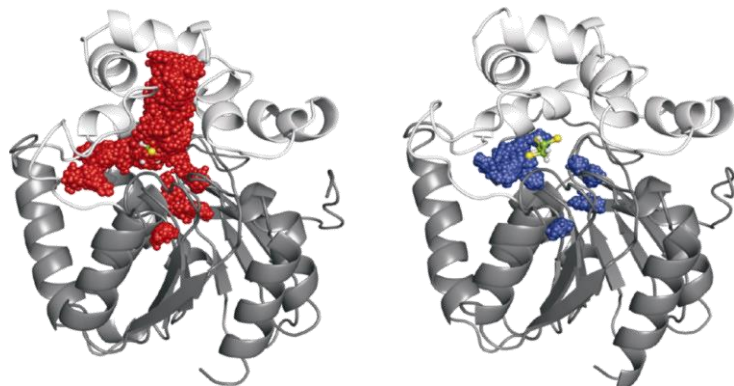
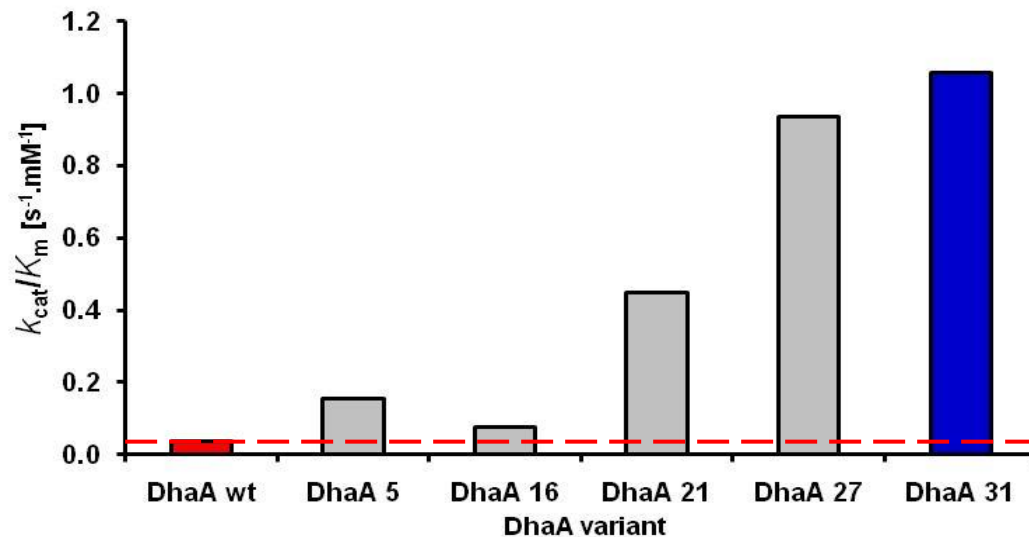


Example of semi-rational design

- ❑ conversion of 1,2,3-trichloropropane by DhaA from *Rhodococcus erythropolis* Y2
- ❑ **DIRECTED EVOLUTION** - importance of access pathways
- ❑ **SEMI-RATIONAL DESIGN** - hot spots in access tunnels
- ❑ library of **5,300 clones** screened



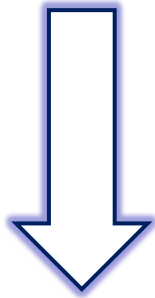
Example of semi-rational design



Experimental throughput is critical

STANDARD DESIGN

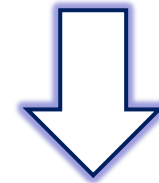
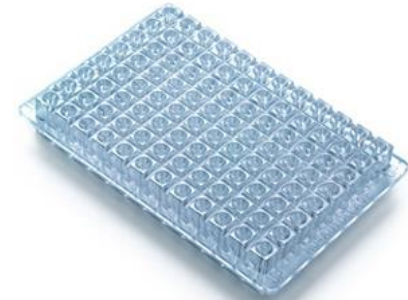
- random mutagenesis (2-3 positions)
- library of 10^4 clones



ADVANCED DESIGN

- random mutagenesis (5-7 positions)
- library of $>10^6$ clones

volume: $100 \mu\text{L}$
assays/day: 10^3

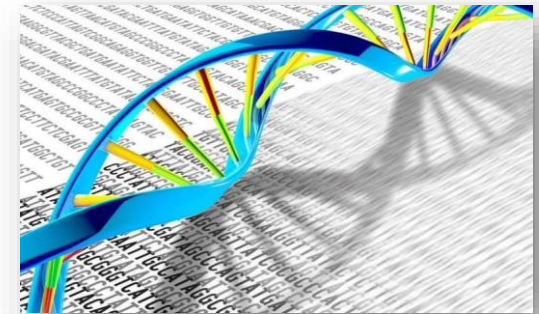
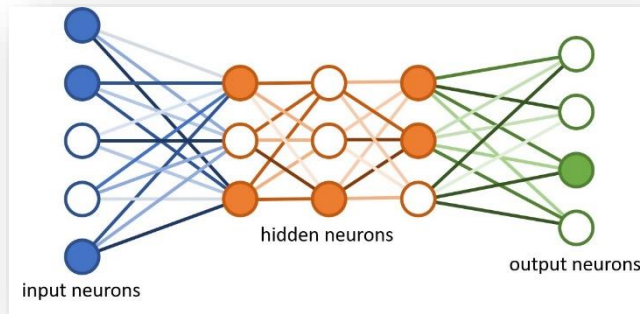
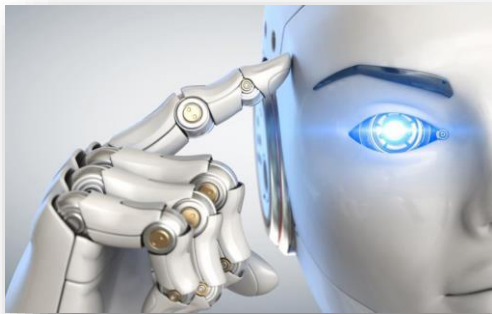


volume: 10 pL
assays/day: 10^7

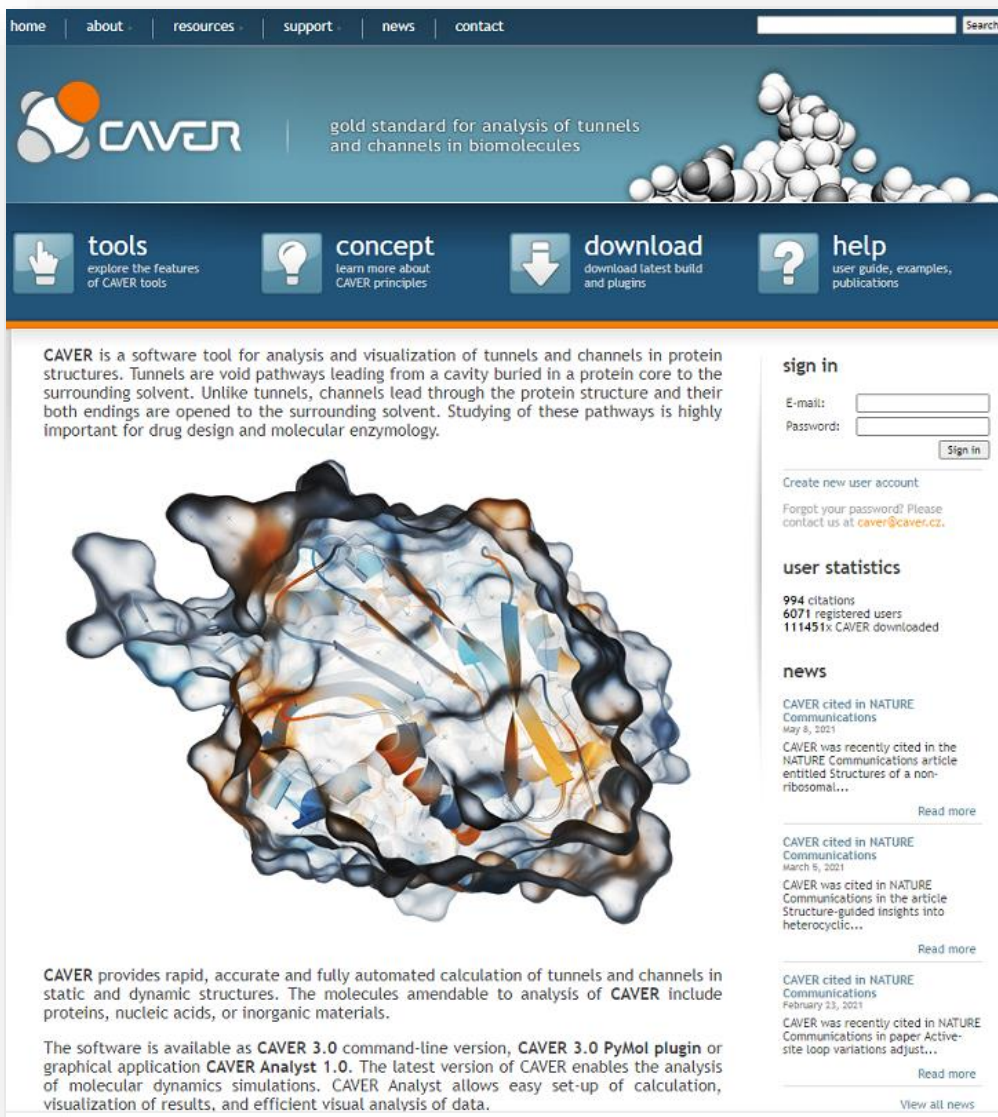


AI in Biology, Chemistry, and Bioengineering Bi9680En

- Období: podzim
- Rozsah: přednáška 2 hodiny/týden
- Vyučující: Dr. Stanislav Mazurenko
- Osnova:
 - modern bio-challenges: drug design, DNA interpretation, protein engineering
 - types of AI algorithms and workflow for designing predictors
 - clustering algorithms, random forests, artificial neural networks
 - features, databases, and predictors used in applications



Tools for protein engineering



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CAVER gold standard for analysis of tunnels and channels in biomolecules

tools explore the features of CAVER tools

concept learn more about CAVER principles

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help user guide, examples, publications

CAVER is a software tool for analysis and visualization of tunnels and channels in protein structures. Tunnels are void pathways leading from a cavity buried in a protein core to the surrounding solvent. Unlike tunnels, channels lead through the protein structure and their both endings are opened to the surrounding solvent. Studying of these pathways is highly important for drug design and molecular enzymology.

CAVER provides rapid, accurate and fully automated calculation of tunnels and channels in static and dynamic structures. The molecules amenable to analysis of CAVER include proteins, nucleic acids, or inorganic materials.

The software is available as CAVER 3.0 command-line version, CAVER 3.0 PyMol plugin or graphical application CAVER Analyst 1.0. The latest version of CAVER enables the analysis of molecular dynamics simulations. CAVER Analyst allows easy set-up of calculation, visualization of results, and efficient visual analysis of data.

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Nucleic Acids Res. 47: W414-W422 (2019)



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Nucleic Acids Res. 45, W393-W399 (2017)



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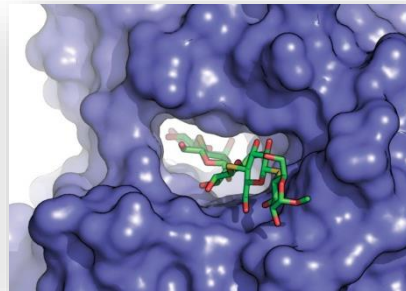


Bioinformatics 37, 23-28 (2021)

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Proteinové inženýrství Bi7410

- Období: jaro
- Rozsah: přednáška 1 hodina/týden
- Vyučující: doc. Radka Chaloupková, Ph.D.
- Osnova:
 - **strukturně-funkční vztahy proteinů**
 - **metody exprese a purifikace rekombinantních proteinů**
 - **metody strukturní a funkční analýzy proteinů**
 - **racionální design, semi-racionální design a řízená evoluce**
 - **příklady využití proteinového inženýrství**



Reading

- ❑ Lutz, S. 2010: **Beyond directed evolution - semi-rational protein engineering and design**. *Curr Opin Biotechnol.* 21(6): 734–743
- ❑ *Computational enzyme redesign and Computational de novo enzyme design (page 5-7)*



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Beyond directed evolution - semi-rational protein engineering and design

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Department of Chemistry, Emory University, 1515 Dickey Drive, Atlanta, GA, 30322

Abstract

Over the last two decades, directed evolution has transformed the field of protein engineering. The advances in understanding protein structure and function, in no insignificant part a result of directed evolution studies, are increasingly empowering scientists and engineers to devise more effective methods for manipulating and tailoring biocatalysts. Abandoning large combinatorial libraries, the