LOSCHMIDT LABORATORIES

PROTEIN ENGINEERING

10. EXAMPLE OF UTILIZING PROTEIN ENGINEERING TO ENHANCE ENZYME STABILITY

Loschmidt Laboratories
Department of Experimental Biology
Masaryk University, Brno

Outline

- Process design criteria
- Engineering enzyme stability and resistance to an organic cosolvent by modification of residues in the access tunnel
  - motivation
  - aims
  - results
  - conclusions
- Method of protein stabilization – patent application

Process design criteria

- higher activity at process conditions
- **increased process stability**
- **increased thermostability** to run at higher temperatures
- **stability to organic solvents**
- absence of substrate and/or product inhibition
- increased selectivity (enantio-, regio-, chemo-)
- accept new substrate
- catalyse new reactions

Motivation

- **organic cosolvents** can have a **positive effect on catalysis**
  - improving substrate solubility
  - alteration substrate specificity and enantioselectivity
  - suppression of water-induced side reactions
- **higher concentration of organic co-solvents** usually cause **protein denaturation**

Aims

- to **identify mutations influencing stability** of haloalkane dehalogenases in organic cosolvent
- to **construct haloalkane dehalogenase with improved stability** in buffer containing DMSO

Methods

- **error-prone PCR** (epPCR) by Taq polymerase, $MnCl_2$
- **screening** by pH indicator assay in MTPs with 42 - 52% DMSO
  phenol red: red ⟷ yellow (pH lower than 6.6)
- **purification** by affinity chromatography
- **thermodynamic stability and structural characterization**
  by circular dichroism, fluorescence spectroscopy, differential scanning calorimetry and X-ray crystallography
- **functional characterization and kinetic stability**
  by activity assay (Iwasaki method) and steady-state kinetics

Methods

- thermodynamic x kinetic stability

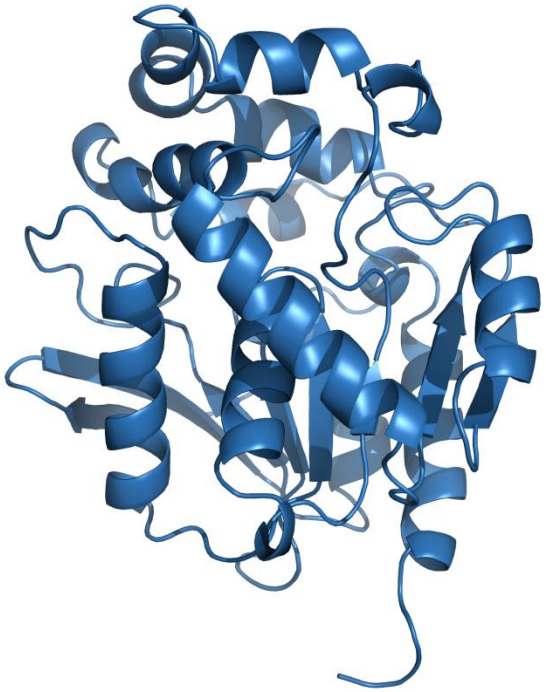
Definitions of various stability parameters.

Measure	Symbol	Type of stability	Definition
Free energy of unfolding	ΔG_u	Thermodynamic	Change in Gibbs free energy going from the folded to unfolded state
Melting temperature	T_m	Thermodynamic	The temperature at which half of the protein is in the unfolded state
Unfolding equilibrium constant	K_u	Thermodynamic	The concentration of unfolded species divided by the concentration of folded species
Half-concentration	$C_{1/2}$	Thermodynamic	The concentration of denaturant needed to unfold half of the protein (chemical equivalent of T_m)
Observed deactivation rate constant	$k_{d,obs}$	Kinetic	Overall rate constant for going from native to deactivation species
Half-life	$\tau_{1/2}$	Kinetic	Time required for residual activity to be reduced to half
Temperature of half-inactivation	T_{50}	Kinetic	Temperature of incubation to reduce residual activity by half during a defined time period
Optimum temperature	T_{opt}	Kinetic	Temperature leading to highest activity
Total turnover number	TTN	Kinetic	Moles of product produced over the lifetime of the catalyst

Methods

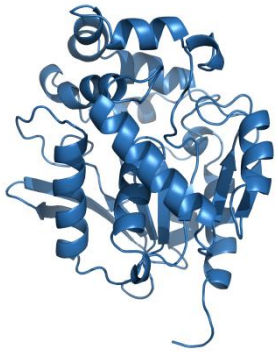
- **site-directed mutagenesis** by QuikChange
- **gene synthesis**
- **saturation mutagenesis** by inverse PCR using a synthetic oligonucleotide with one degenerated NNK codon
- **molecular basis of resistance to organic cosolvent** by molecular dynamics simulations in 40% DMSO

Studied HLD



DhaA
from *Rhodococcus rhodochrous*

Directed evolution

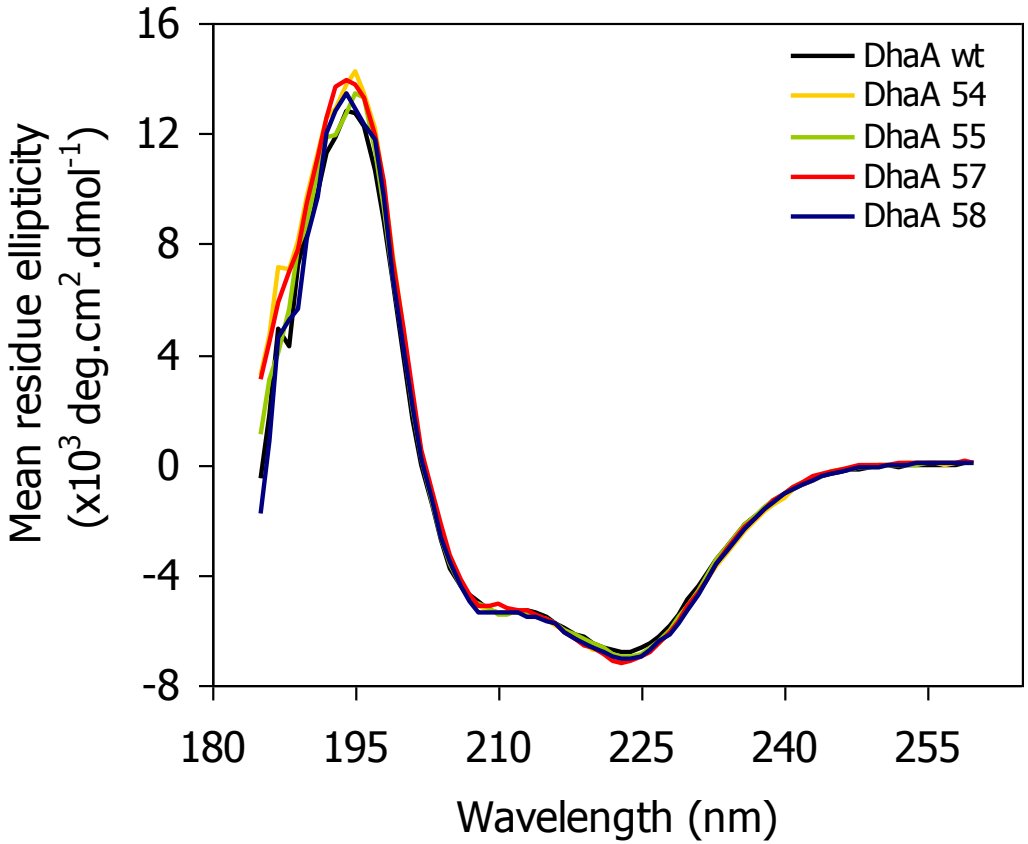


DhaA

epPCR

4 positive hits

Mutant resistant to DMSO



T_m = 50.4 C

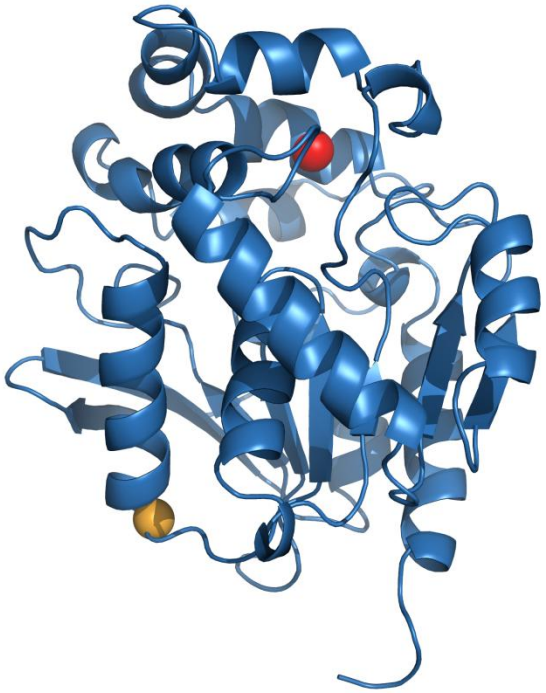
T_m = 50.6 °C

T_m = 52.6 °C

T_m = 55.9 °C

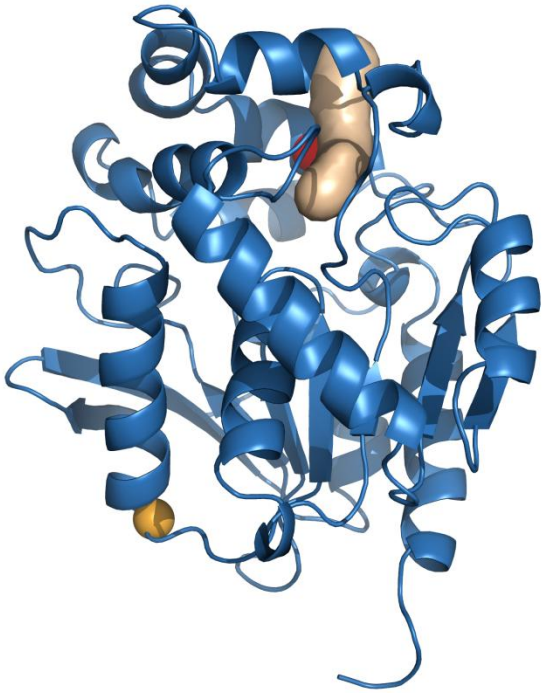
T_m = 52.5 °C

Mutant resistant to DMSO



DhaA 57

Mutant resistant to DMSO



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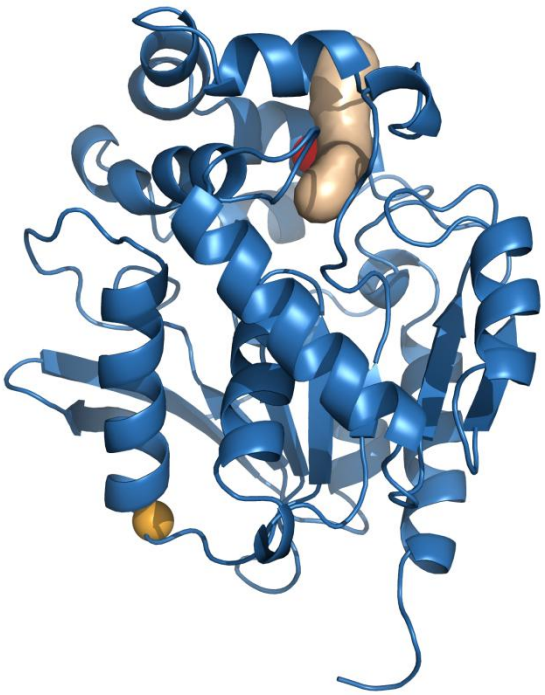

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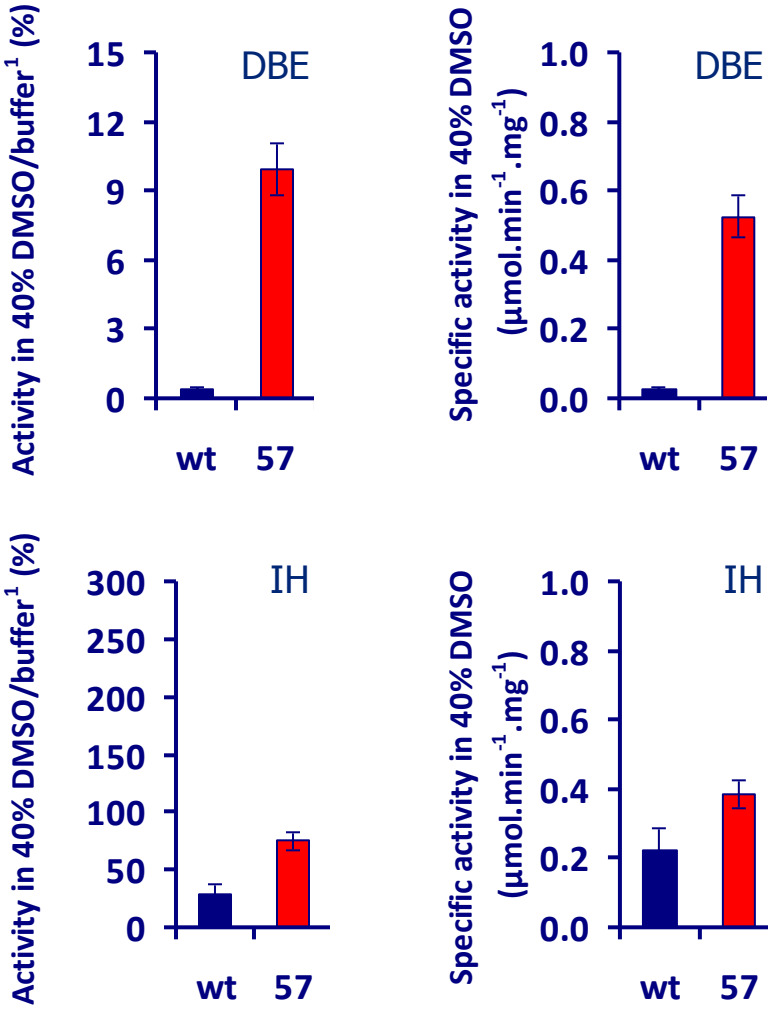
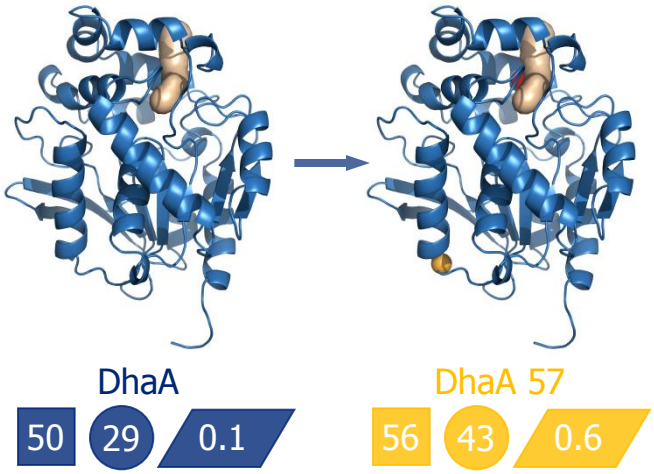


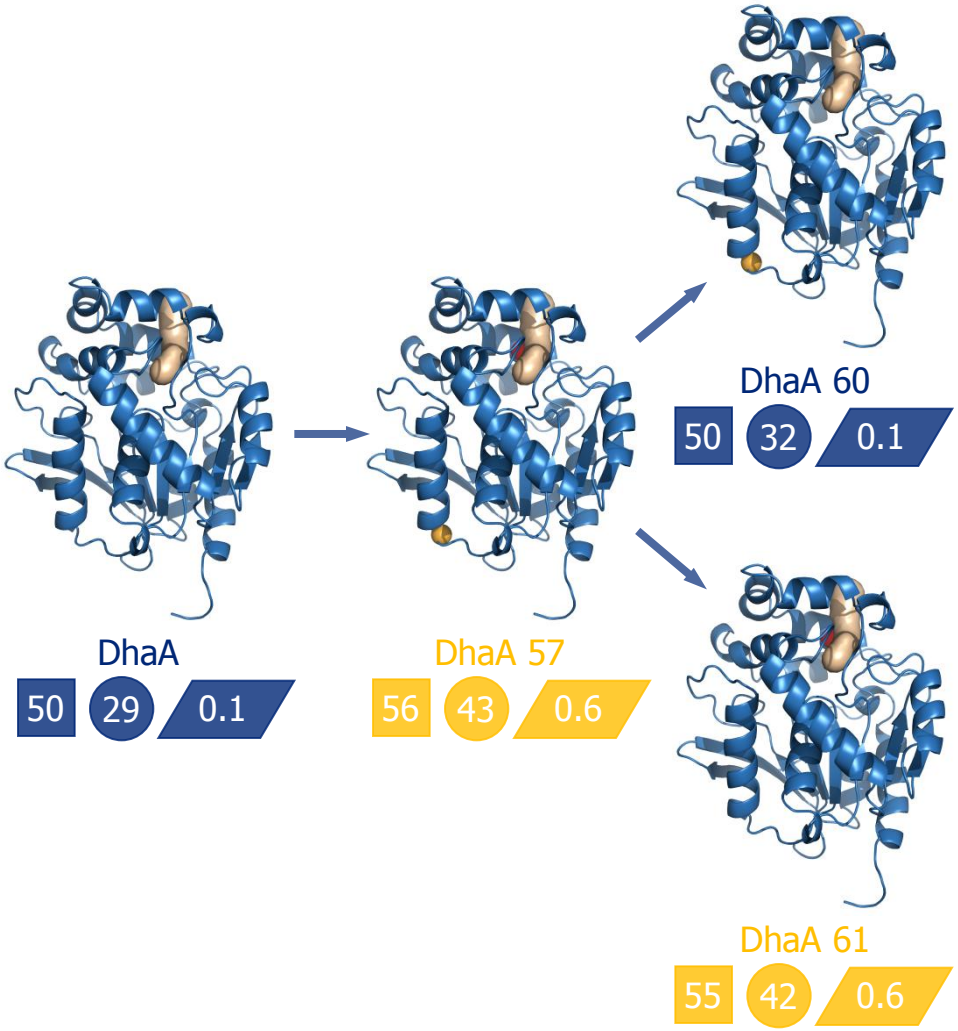
DBE – 1,2 dibromoethane; IH – iodohexane



melting temperature in buffer (°C)

half-concentration of DMSO (%)

half-life in 40% DMSO at 37 °C (h)



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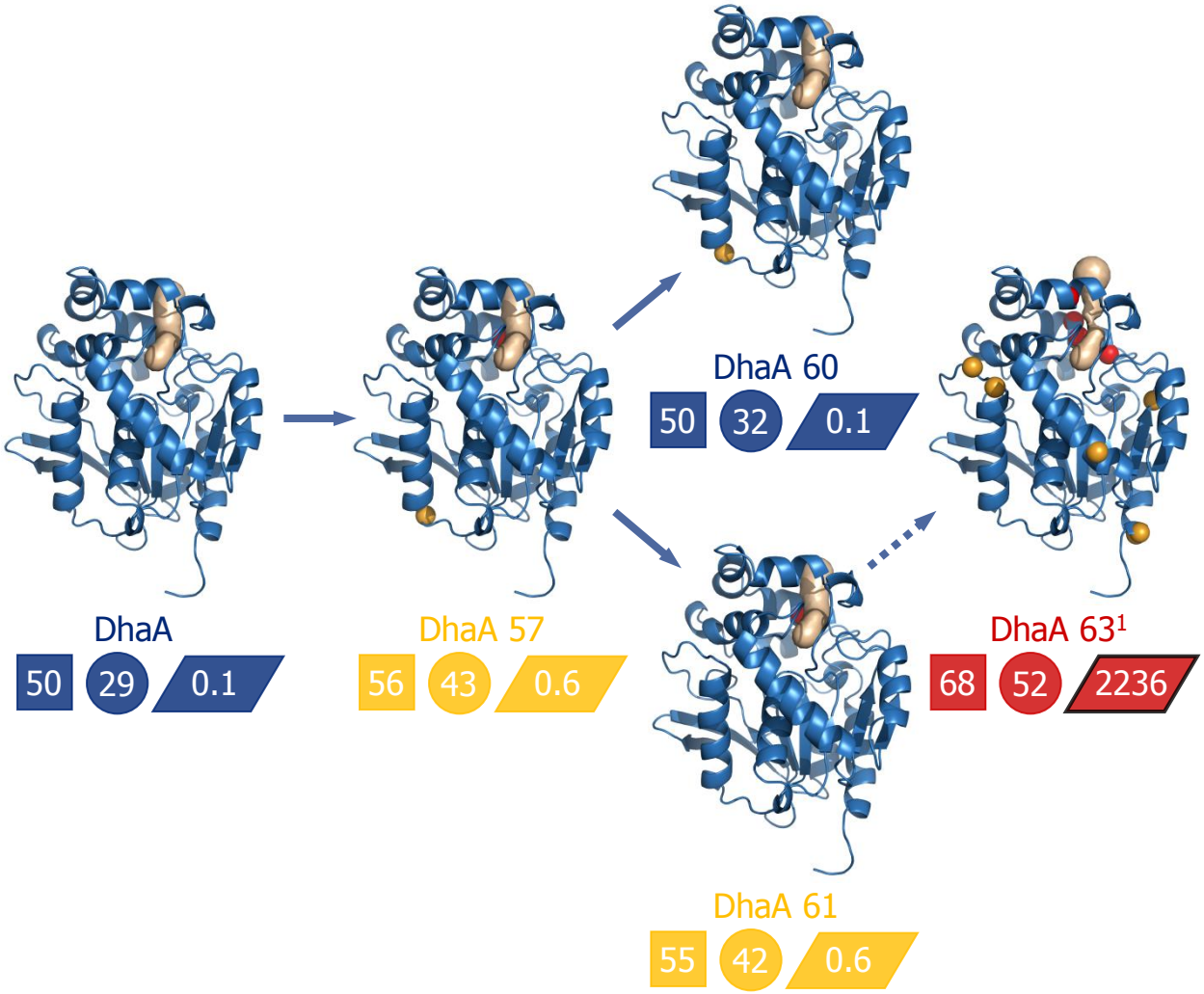

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[1]Gray, K.A. et al.: *Adv. Synth. Catal.* 343, 607-617 (2001)

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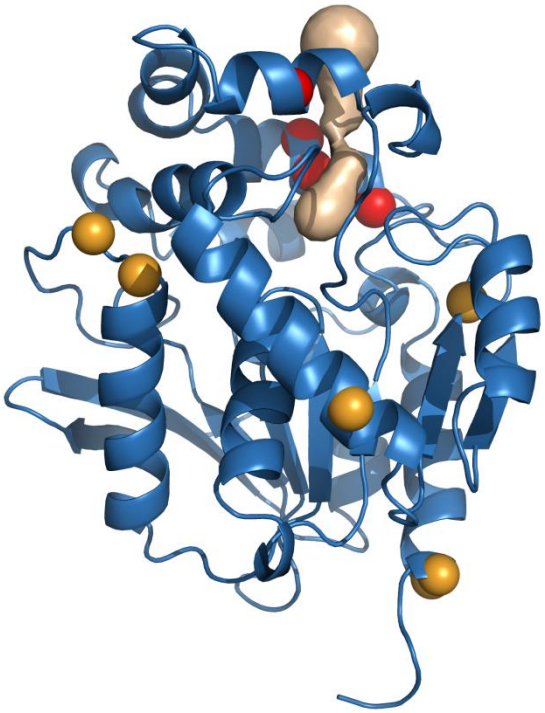
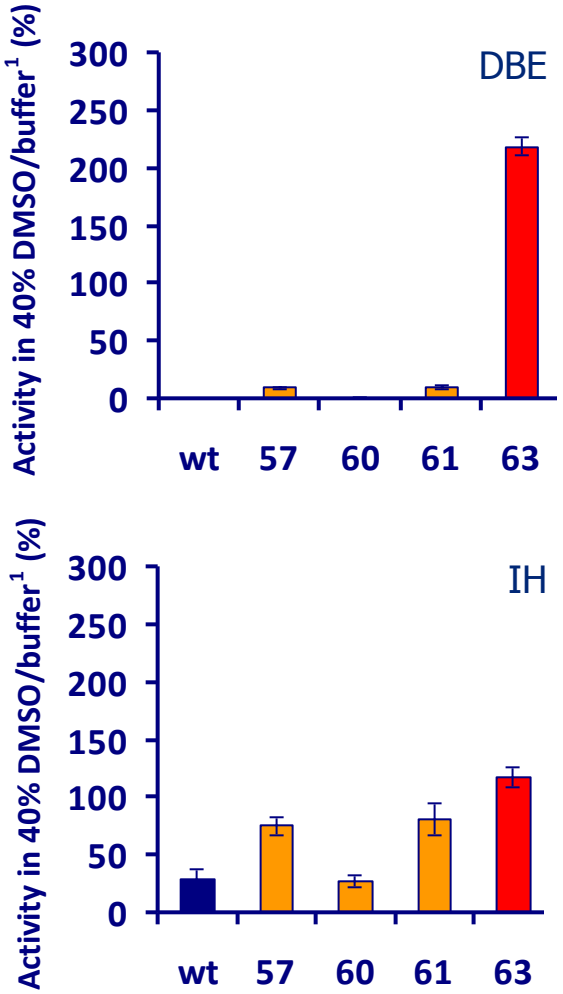


DhaA 63



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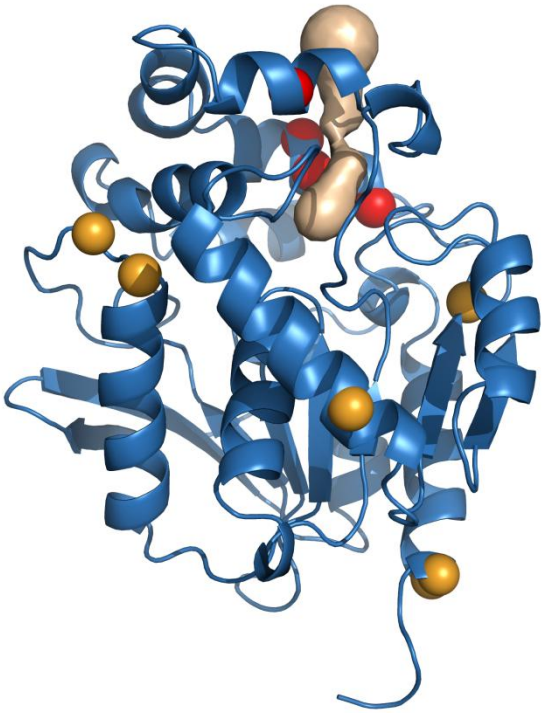

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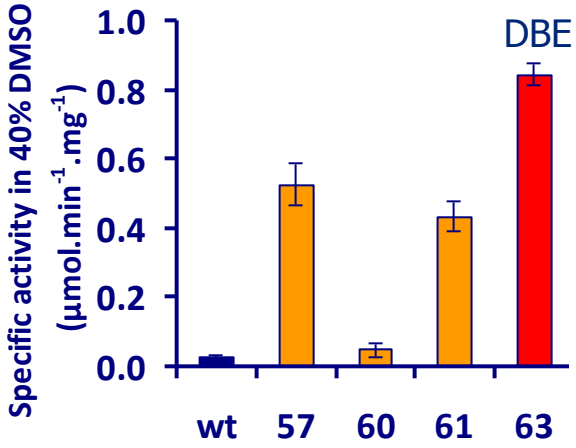

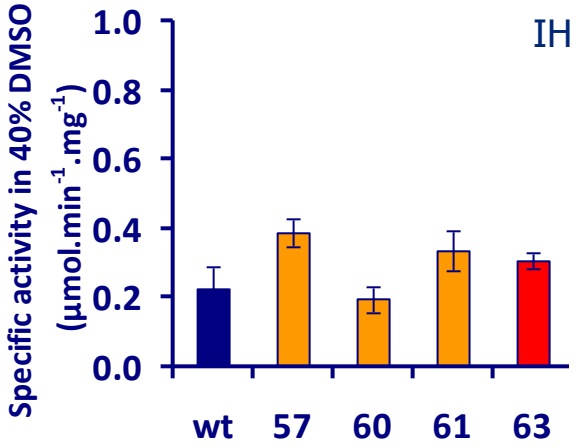

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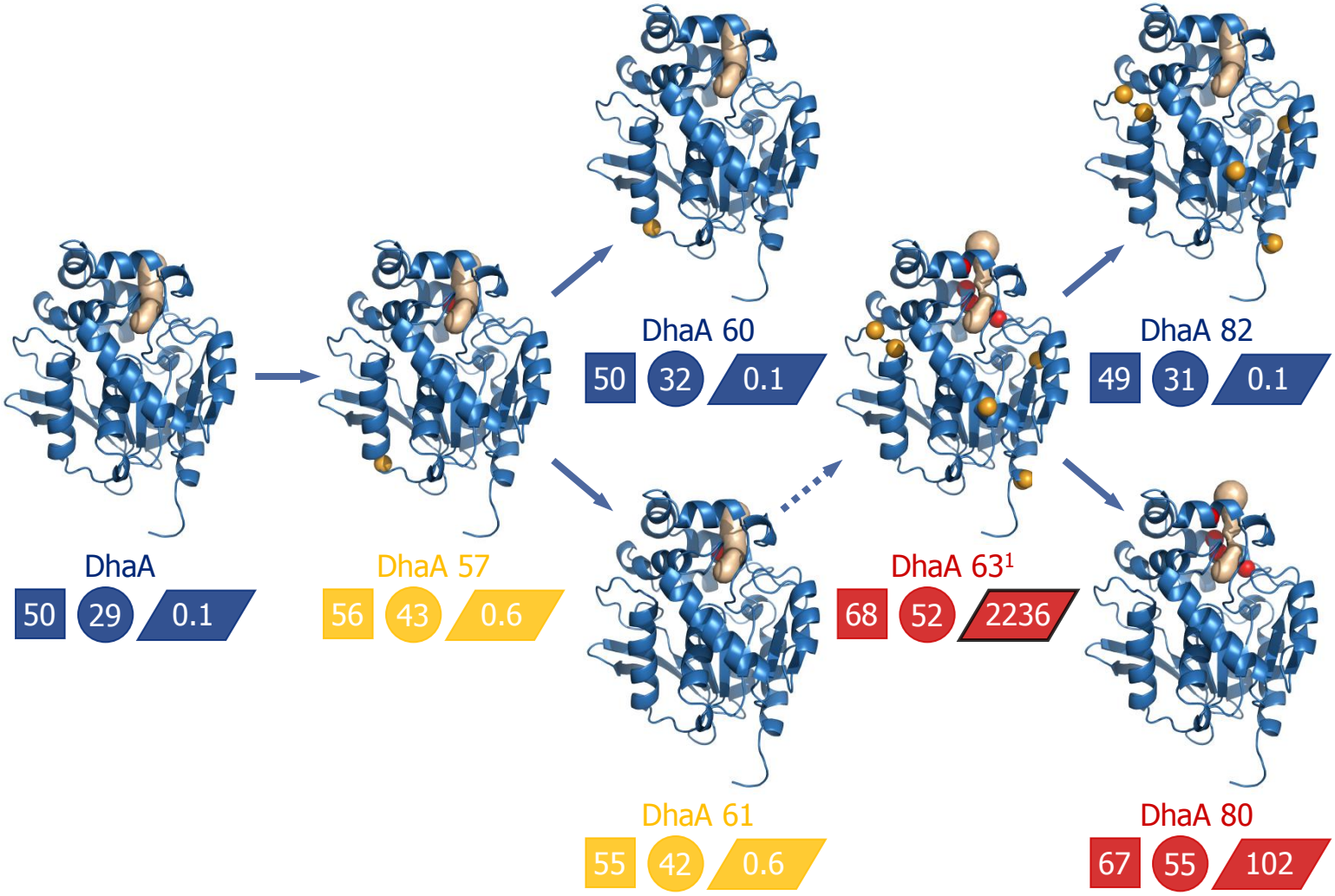

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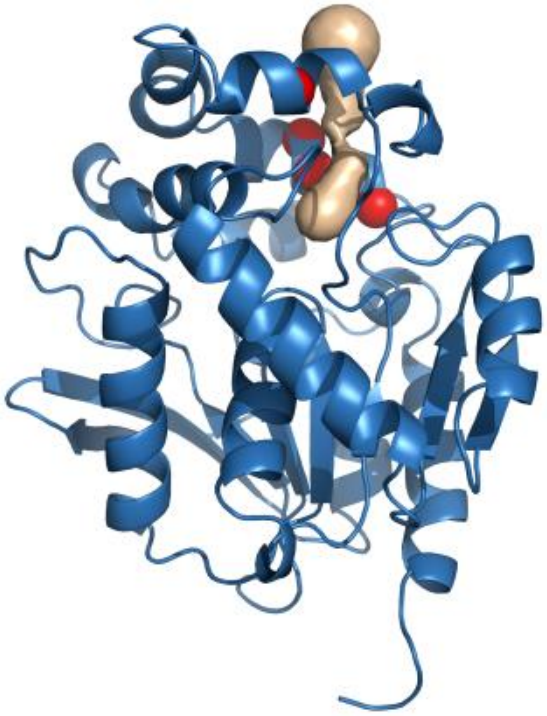
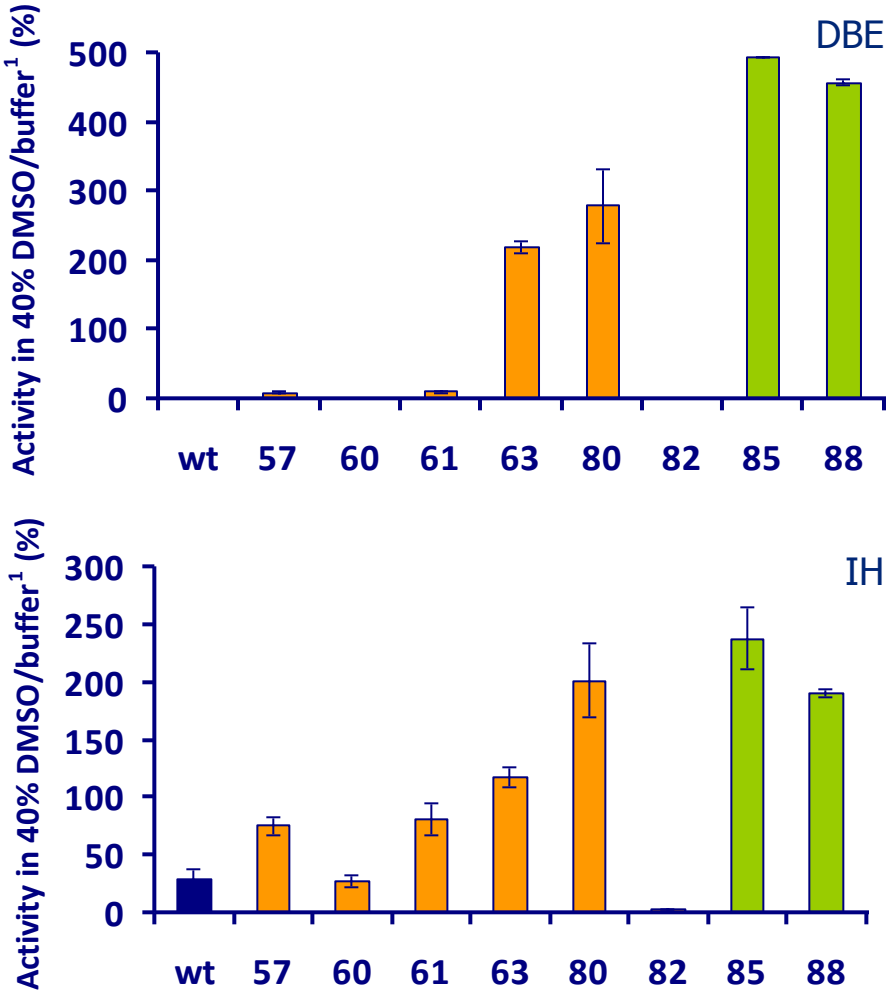


DhaA 85, DhaA 88



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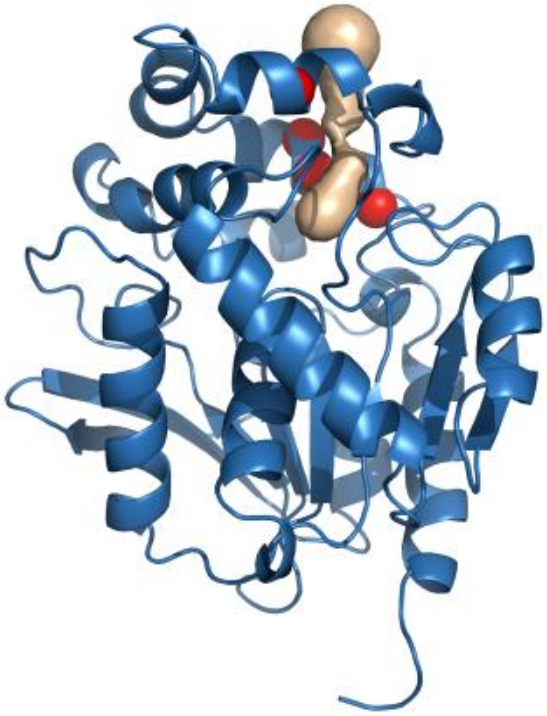

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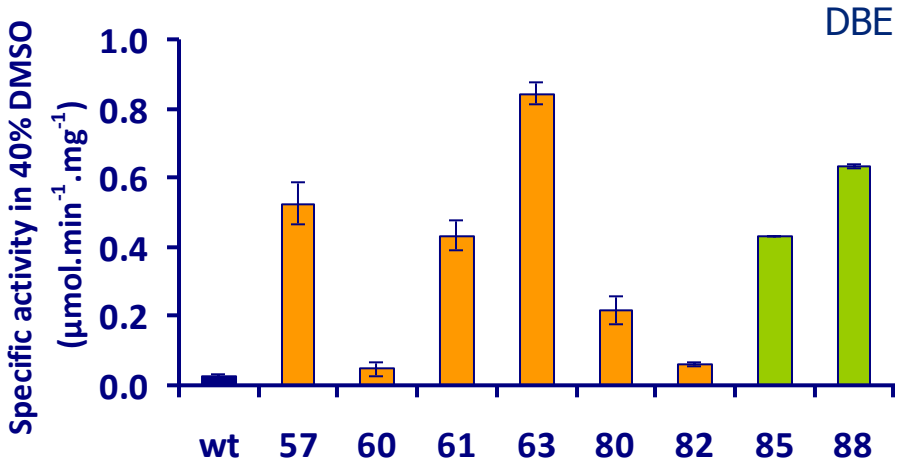

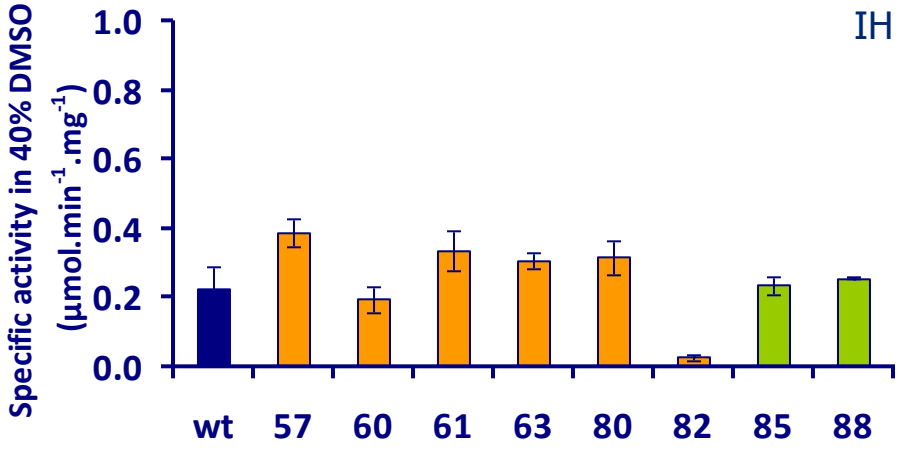

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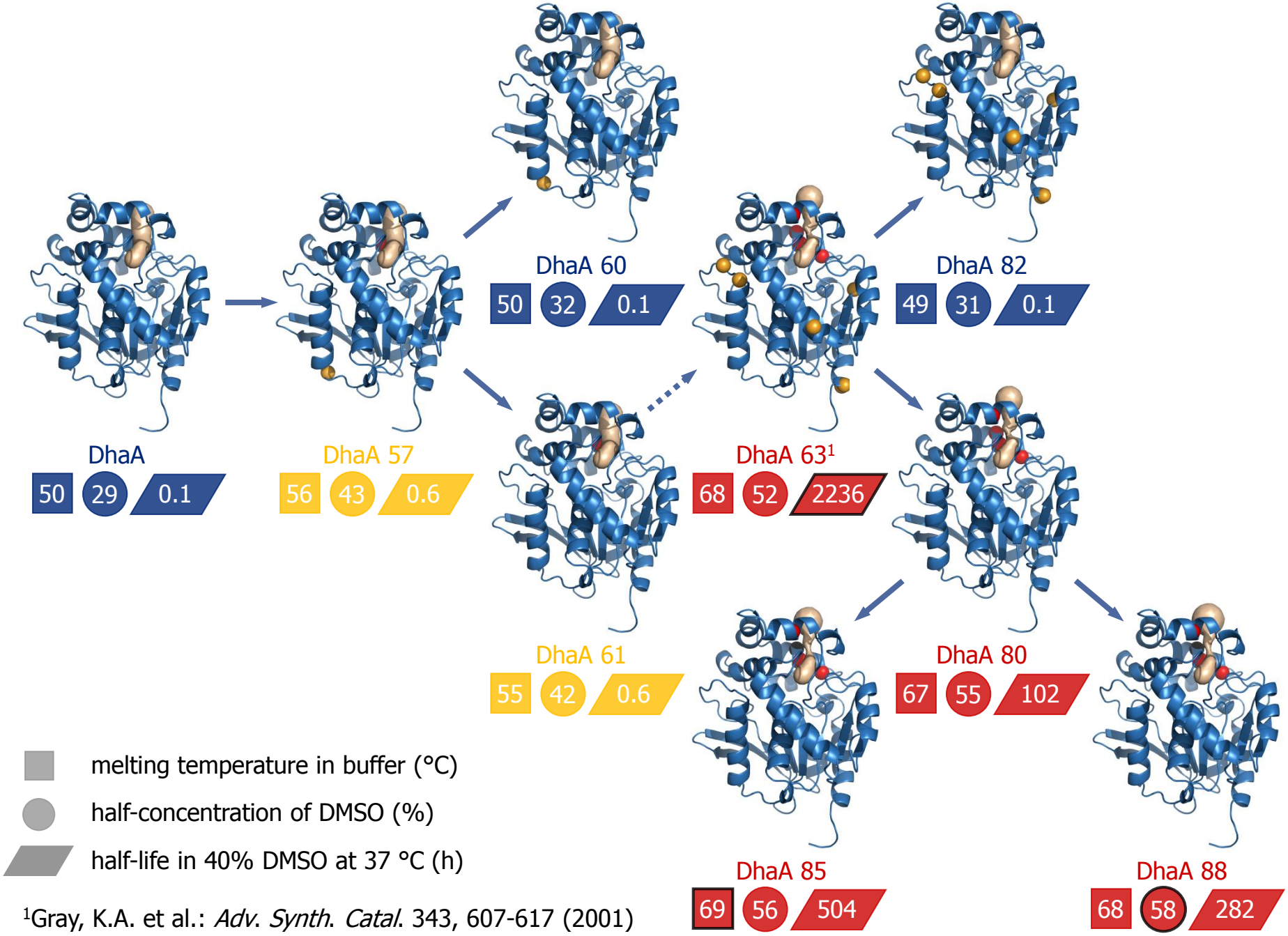
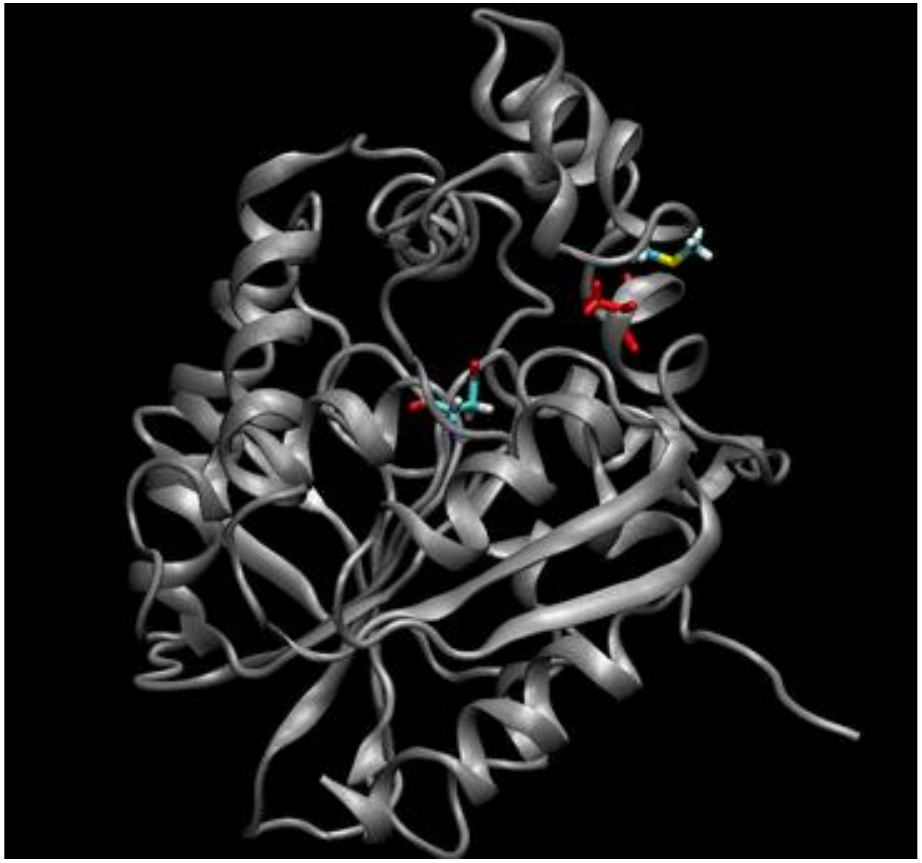


[1]Gray, K.A. et al.: *Adv. Synth. Catal.* 343, 607-617 (2001)

Mutant resistant to DMSO

DhaA wt



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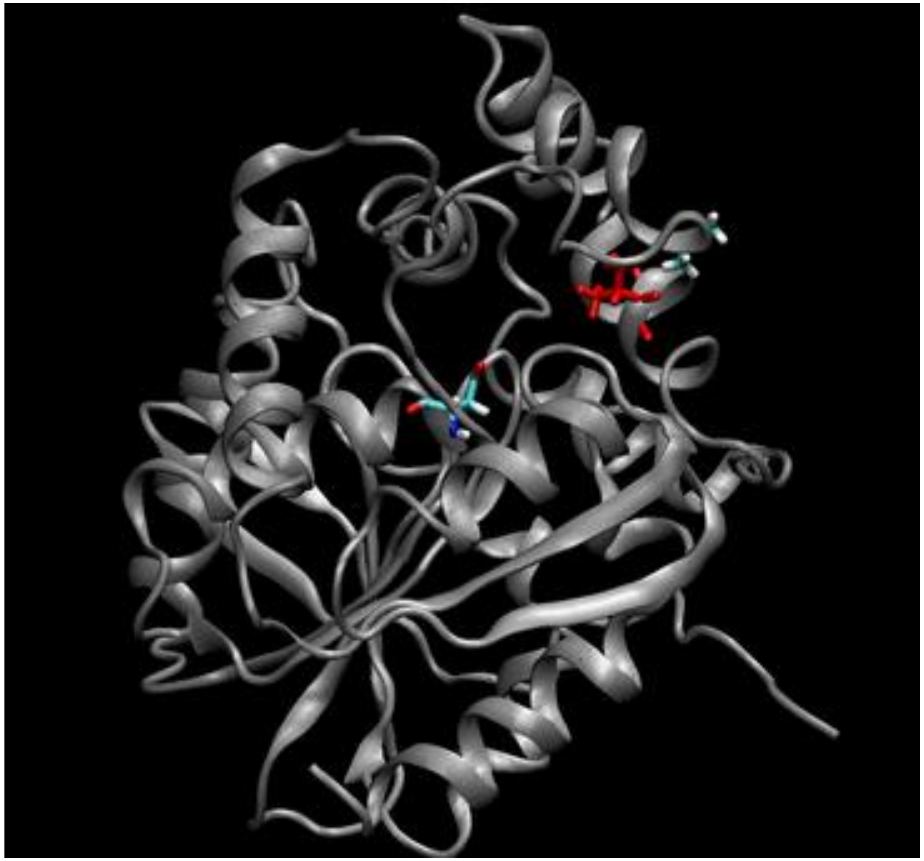
DhaA 57



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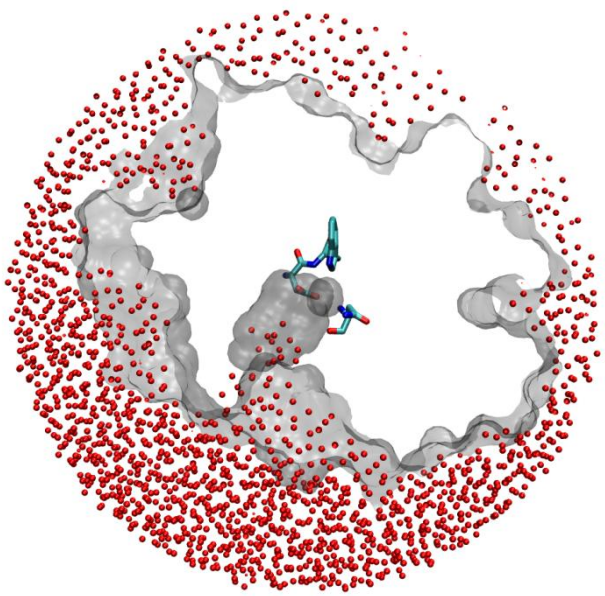
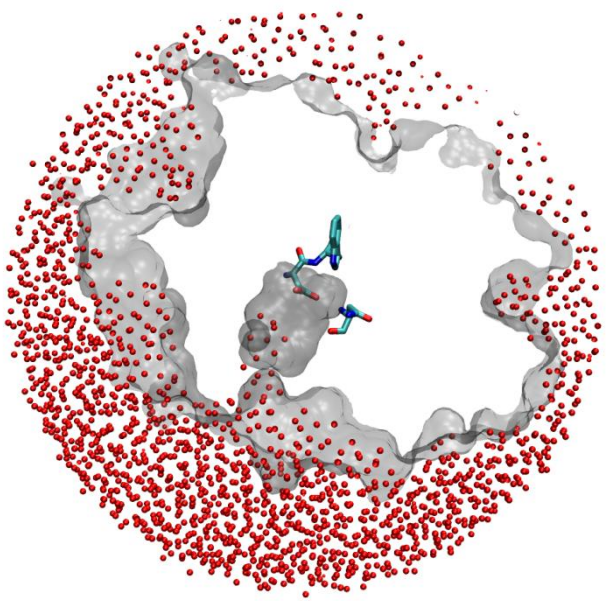


DhaA wt



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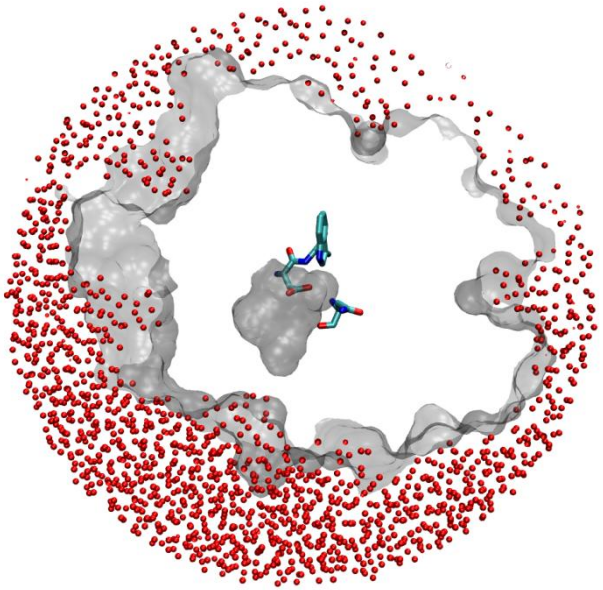


DhaA 80

Conclusion

- **resistance** towards organic cosolvents **correlates with thermostability**
- mutations lining access tunnel modulate **occupancy** of active site by solvent and can **stabilize** protein
- robust catalysts were developed:
  4 point mutations, T_m ↑ **19 C**, $T_{1/2}$ (40 % DMSO) **min** → **days**
- **engineering of access tunnels represents novel strategy for engineering of robust catalysts**

Conclusion

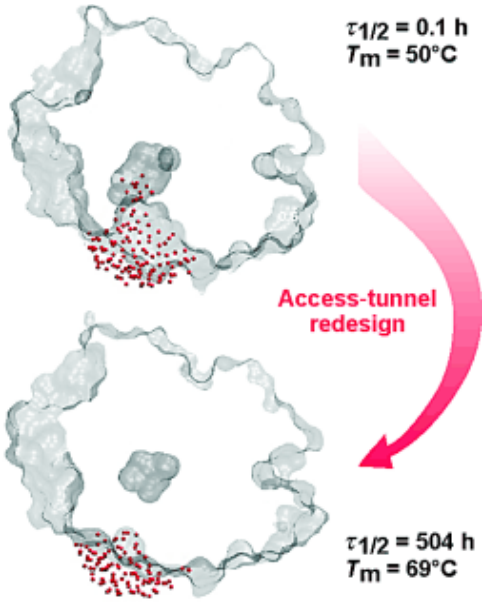
Angewandte International Edition Chemie

Protein Stability

DOI: 10.1002/anie.201206708

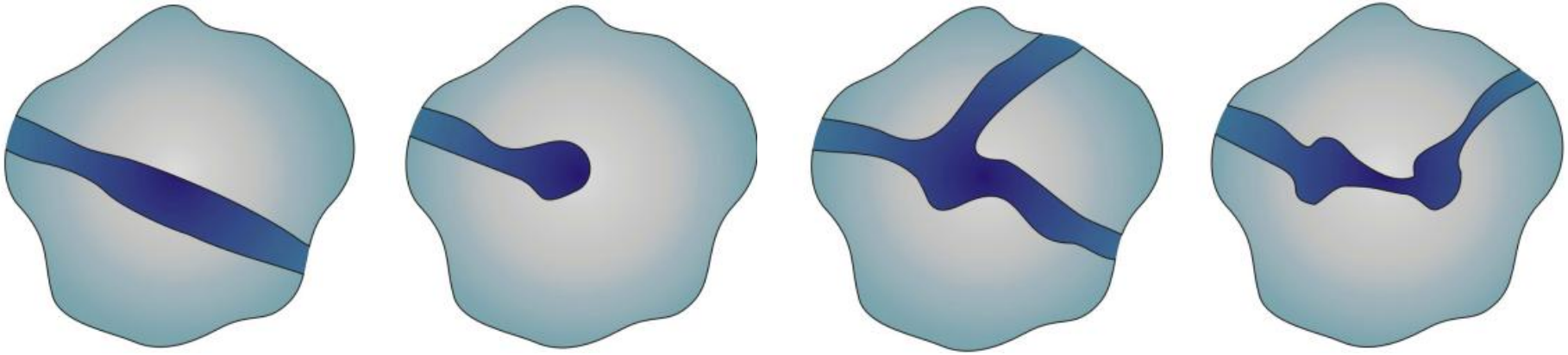
Engineering Enzyme Stability and Resistance to an Organic Cosolvent by Modification of Residues in the Access Tunnel**

*Tana Koudelakova, Radka Chaloupkova, Jan Brezovsky, Zbynek Prokop, Eva Sebestova, Martin Hesseler, Morteza Khabiri, Maryia Plevaka, Daryna Kulik, Ivana Kuta Smatanova, Pavlina Rezacova, Rudiger Ettrich, Uwe T. Bornscheuer, and Jiri Damborsky**



Method of protein stabilization

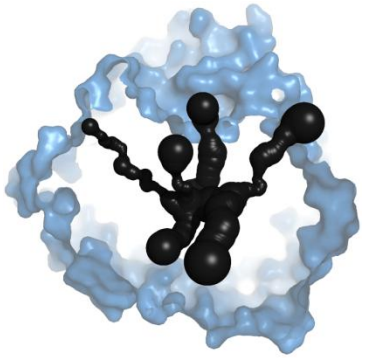
- method for modification of the access routes in order to achieve better stability of protein towards temperature and solvents
- definition of the access routes: channel x tunnel



Method of protein stabilization

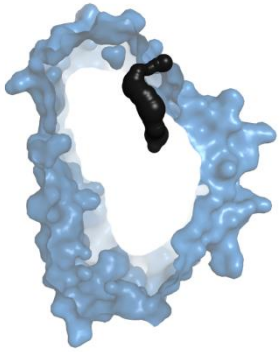
- method for modification of the access routes in order to achieve better stability of protein towards temperature and solvents
- definition of the access routes: channel x tunnel
- general concept, tunnels found in all enzyme classes

1. OXIDOREDUKTASES



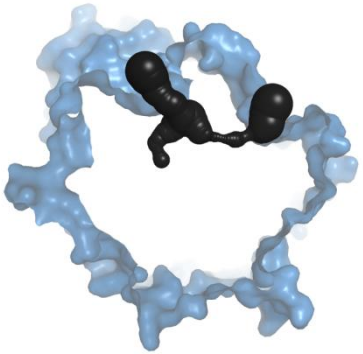
Cytochrome CYP3A4
EC 1.1.3.6

2. TRANSFERASES



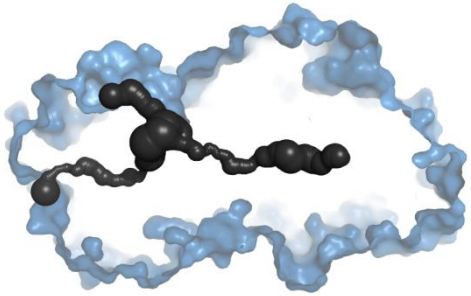
Chalcone synthase
EC 2.3.1.74

3. HYDROLASES



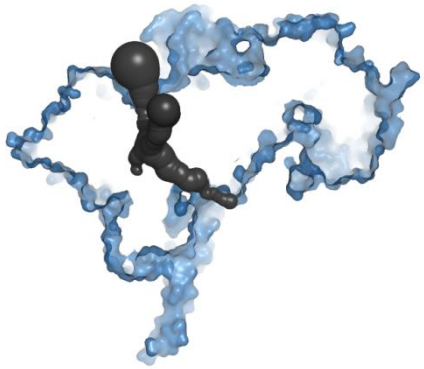
Acetylocholinesterase
EC 3.1.1.7

4. LYASES



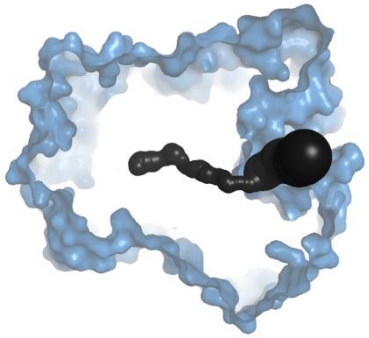
Tryptophan synthase
EC 4.2.1.20

5. ISOMERASES



Methylmalonyl-CoA mutase
EC 5.4.99.2

6. LIGASES



Asparagine synthetase
EC 6.3.1.1

Method of protein stabilization

- procedure of protein stabilization

  - identification the amino acids lining access routes based on knowledge of structure (CAVER, HotSpot Wizard)

  - modification of selected amino acids „hot spots“ (site-directed mutagenesis, random mutagenesis)

  - analysis of constructed variants/libraries, assessment of the result of modification

- rational focused mutagenesis based on detailed knowledge of structure and function

  - creation of small focused “smart” libraries

  - increase likelihood of beneficially modifying property

Method of protein stabilization

- modification of shape and physico-chemical properties of tunnels

  - selective discrimination between the molecules of a substrate/product and undesired solvent molecules inside the access routes

  - strengthening of hydrophobic interactions within the tunnel

  - thermostability enhancement

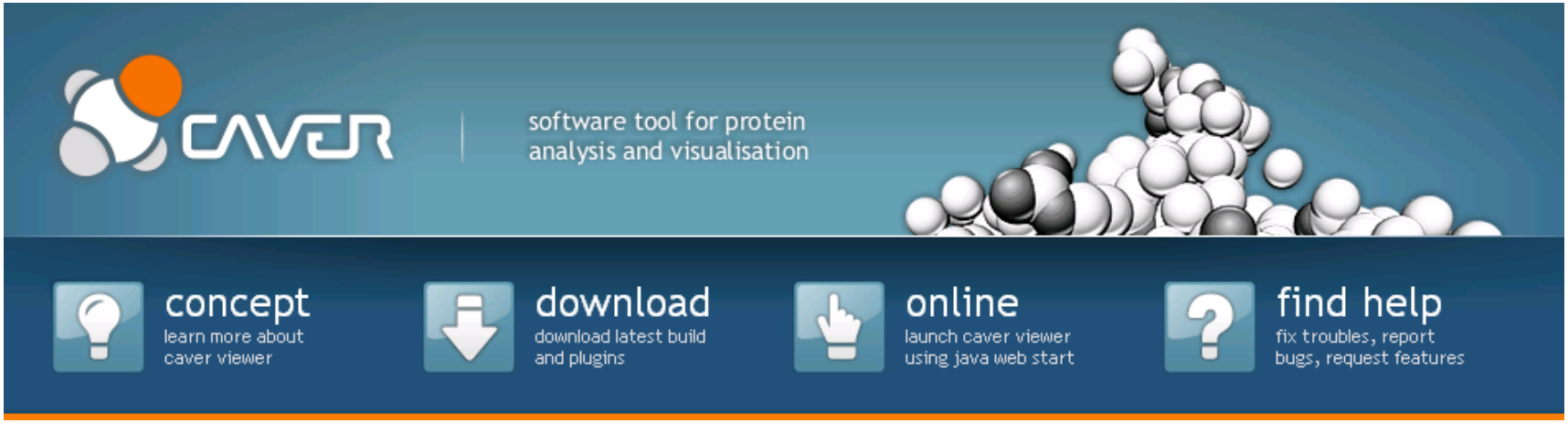
- high thermostability and resistance against organic cosolvents = required process design criteria

- invention describing the method of stabilization patented

Damborsky, J., Prokop, Z., Koudelakova, T., Stepankova, V., Chaloupkova, R., Chovancova, E., Gora, A., Brezovsky, J., 2011: Method of thermostabilization of a protein and/or stabilization towards organic solvents. Patent PV 2011-680.

Method of protein stabilization

- identification of tunnels – CAVER[1]



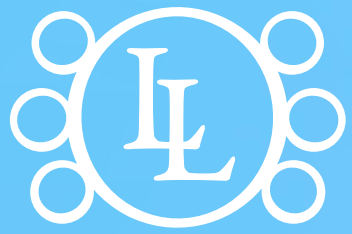
www.caver.cz

[1]Chovancova E. *et al.,* 2012, PLoS Comp. Biol. 8: e1002708

Helpful references

- Koudelakova, T. et al. (2013) Engineering enzyme stability and resistance to an organic cosolvent by modification of residues in the access tunnel, Angew. Chem. Int. Ed. 52: 1959-1963
- Gray, K.A. (2001) Rapid evolution of reversible denaturation and elevated melting in a microbial haloalkane dehalogenase, Adv. Synth. Catal. 343: 607-617
- Chovancova, E. (2012) CAVER 3.0: A Tool for Analysis of Transport Pathways in Dynamic Protein Structures, PLoS Comput. Biol. 8: e1002708

- **QUESTIONS?**



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