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IN THE ENVIRONMENT (RECETOX)

STRUCTURE, FUNCTION AND ENGINEERING OF HALOALKANE DEHALOGENASES

Habilitation thesis

Mgr. Radka Chaloupková, Ph.D.

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Poděkování

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Author: Mgr. Radka Chaloupková, Ph.D.
Loschmidt Laboratories
Department of Experimental Biology
and Research Centre for Toxic Compounds in the Environment
Faculty of Science, Masaryk University

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Autor: Mgr. Radka Chaloupková, Ph.D.
Loschmidtovy laboratoře
Ústav experimentální biologie
a Centrum pro výzkum toxických látek v prostředí
Přírodovědecká fakulta, Masarykova univerzita

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Mým dědům do nebe

Napadl v červnu sníh na ulici, štěkali ptáci, cvrlikali psíci.
Létaly krávy na modré louce, na nebi pělo zelené slunce.
Hnízdečka v květech motýli vili, trvalo všecko maličkou chvíli.
Viděl jsem divy čarovné moci, když jsem měl právě přivřené oči.
Když jsem se rozhléd, všecko se skrylo, na světě zase vše jak dřív bylo.
Všecko se pěkně děje a točí… Od těch chvil často přivírám oči.
(**Zázraky a divy, Julian Tuwim**)

CONTENT

SUMMARY

Haloalkane dehalogenases make up an important class of enzymes that cleave carbon-halogen bond in halogenated aliphatic compounds. There is a growing interest in these enzymes due to their potential use in environmental applications and biocatalysis. Utilization of enzymes in industrial processes requires a number of criteria to be fulfilled: high activity and stability under process conditions, appropriate substrate specificity, supressed substrate/product inhibition, and sufficient enantioselectivity. Understanding of the structural determinants of activity, substrate specificity and enantioselectivity of haloalkane dehalogenases will therefore facilitate the attempts to modify these enzymes for practical applications.

The habilitation thesis summarizes *current* scientific *knowledge* on structure, function and engineering of haloalkane dehalogenase enzyme family. The thesis covers the introduction of the principles of methods frequently used for analysis of structure-function relationships of haloalkane dehalogenases, including circular dichroism spectroscopy, X-ray crystallography, isothermal titration calorimetry, stopped-flow, and rapid quench-flow methods. Overview of various strategies employed for modification of haloalkane dehalogenase catalytic properties is also provided. Each section is supplemented by the practical examples extracted from the authors' publications. These publications represent milestones in the understanding of haloalkane dehalogenase catalysis and were published in high ranking journals. Finally, potential future research directions, such as analysis of hydration and flexibility of the enzymes, identification and characterization of novel family members, development of high-throughput screening, and identification of biological function of haloalkane dehalogenases are discussed.

INTRODUCTION

1. Haloalkane dehalogenases - general description, origin and potential application

The first part of the introduction section is focused on a general description of haloalkane dehalogenases that represent the main subject of the studies covered by this habilitation thesis. Haloalkane dehalogenases (HLDs, EC 3.8.1.5) are enzymes catalyzing a hydrolytic conversion of halogenated aliphatic alkanes and their derivatives (e.g. haloalcohols, halogenated amides, haloethers and haloesthers) into three reaction products: an alcohol, a halide anion and a proton[1,2]. Individual members of HLD family are able to convert a wide spectrum of chlorinated, brominated and iodinated substrates with a halogen bound to sp^3-hybridised carbon atom[1–3]. There is no evidence for conversion of fluorinated alkanes by HLDs, but fluoroacetate can be hydrolyzed by a dehalogenase enzyme that is structurally and mechanistically related to HLDs[2].

HLDs have drawn considerable interest due to their unique catalytic mechanism and broad substrate specificity. HLDs can be used for biodegradation of toxic, mutagenic, or carcinogenic halogenated compounds,[1,4–6] and biocatalytic production of alcohols[7–11] that can be used as valuable building blocks in organic and pharmaceutical synthesis. Besides, HLDs can be applied in recycling of by-products from chemical processes[12], biosensing of environmental pollutants[13–15], decontamination of warfare agents[16] as well as protein tagging for cell imaging and protein analysis[17,18].

During the past three decades, 24 HLDs have been identified and biochemically characterized[1,19–23]. Most of them are of bacterial origin and were found on a chromosome or a plasmid of various gram-positive and gram-negative bacteria isolated from soil, marine water or clinical samples. The exceptions represent a recently identified HLD from purple sea urchin *Strongylocentrotus purpuratus*[19] and luciferase from sea pansy *Renilla reniformis*[24,25], which sequentially belong to HLD enzyme family, although it does not exhibit dehalogenase activity. A growing number of completed genome sequencing projects disclosed that the genes of putative haloalkane dehalogenases can be found in genomes of more than 350 bacterial species, 16 eukaryotic organisms and 5 archaea organisms (Vanacek et al., unpublished data). Phylogenetic analysis of HLDs and their putative relatives revealed that the enzyme family can be divided into three subfamilies, denoted as HLD-I, HLD-II and HLD-III. These subfamilies differ in the composition of the catalytic pentad and the anatomy of the cap domain[26].

Several HLDs isolated from bacteria inhabiting contaminated soils have been shown to be involved in biodegradation pathways of important environmental pollutants[27–30]. Furthermore, it was found out that various organisms which are not involved in degradation of halogenated compounds also possess genes that encode HLDs. Such HLDs have been isolated from see water[20,21,31–33], obligatory pathogens[31,34–37], plant symbionts[38,39], plant parasites[40] and Siberian permafrost[41]. Biological function of these enzymes remains unknown, but it has been speculated that, for example, HLDs isolated from mycobacterial pathogens could be involved in the detoxification of halogenated compounds produced in a tissue by peroxidases[2]. HLDs isolated from symbiotic rhizobial strains may be important for the interaction and/or communication with plants that produce halogenated compounds as attractants and/or repellents. HLDs from marine environment may play a role of detoxifying enzymes, since a wide range of structurally diverse halogenated compounds is produced by algae and tube worms[3].

2. Structure of HLDs

HLDs structurally belong to the superfamily of α/β-hydrolases[42–44]. This protein family contains > 60 000 proteins[45] of a widely differing phylogenetic origin and catalytic function. The three-

dimensional structure of HLDs is composed of two domains: a main domain and a cap domain. The core of the enzyme, the main domain, is composed of three layers (αβα) – highly conserved central eight-stranded β-pleated sheet with seven parallel strands and one antiparallel strand, which is surrounded by two and four α-helices (Figure 1). The major role of conserved main domain is to provide a scaffold for catalytic residues and the active site cavity. The smaller domain, located between β-strand 6 and α-helix 8, is composed solely of α-helices and is called cap domain. The cap domain covers a top of the main domain, splitting the main domain into the N- and C-terminal part (Figure 1) and shows high variability in its arrangement and composition. The cap domain is believed to determine substrate specificity of HLDs by: (i) nature of the active site residues, (ii) size, shape and flexibility of the active site cavity, and (iii) size, shape, physico-chemical properties and dynamics of access pathways[46–51].

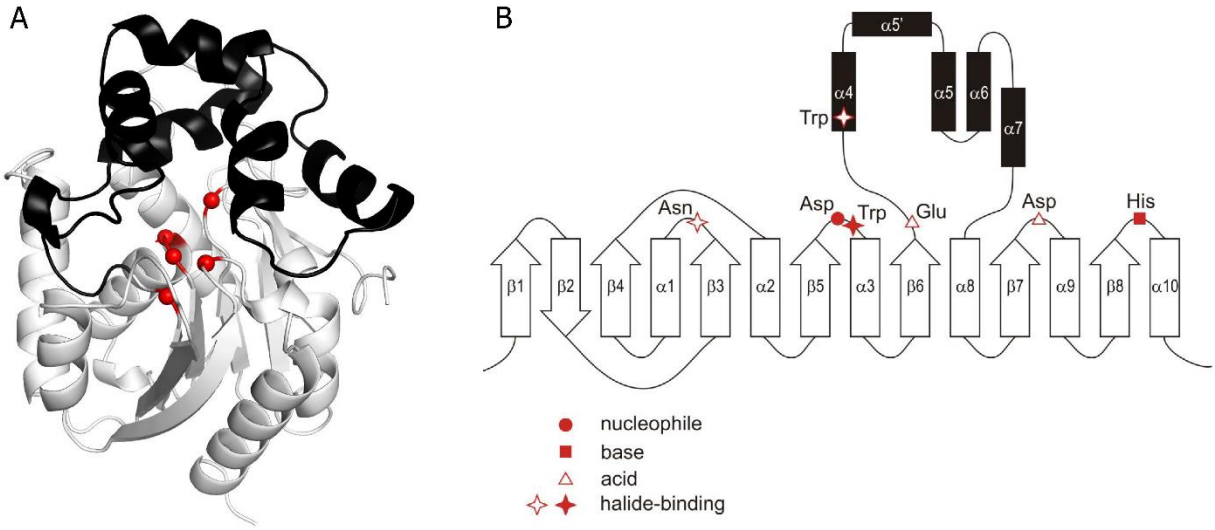


Figure 1. Tertiary structure (A) and general topology (B) of HLDs. The main domain is shown in white and the cap domain is shown in black. The amino acid residues forming the catalytic pentad are represented by red balls and symbols. Nucleophile, catalytic base, and one halide-stabilizing residue are conserved among all HLDs (filled symbols), whereas the catalytic acid and second halide-stabilizing residue differ among HLDs (empty symbols).

Both the main and the cap domain contribute to the formation of buried predominantly hydrophobic active site cavity located at the interface between the domains (Figure 1). The active sites of various HLDs differ in their size and accessibility to the solvent. The buried active sites of HLDs are connected with the protein surface by several access pathways called tunnels. These tunnels represent a very important structural features of HLDs[46]. The size, shape, physico-chemical properties and dynamics of the tunnels are important determinants of stability, catalytic activity, substrate specificity and enantioselectivity in HLDs[52]. Tunnels can contribute to the following steps of HLDs catalytic cycle: (i) access of a substrate, (ii) binding of a substrate, (iii) solvent access, and (iv) release of reaction products. Access tunnels can be either permanent or transient[53] features of HLDs. The permanent tunnels are observable in the ligand-free crystal structures, while the transient tunnels can only be identified in the crystal structures of the protein-ligand complexes or in molecular dynamic simulations.

Without exception, all HLD enzymes comprise five key residues situated inside the active site pocket which are called a catalytic pentad. The catalytic pentad of HLDs is composed of a nucleophile, a

base, a catalytic acid and two halide-stabilising residues. Three catalytic residues are fully conserved among different HLD subfamilies (nucleophilic aspartate located between β-strand 5 and α-helix 3, basic histidine situated after β-strand 8 and halide-stabilising tryptophan placed next to the nucleophilic aspartate)[26], one differs in a position and nature within the main domain (acidic glutamate in the N-terminal part of the main domain or acidic aspartate in the C terminal part of the main domain), and one differs in a nature and interdomain position (second halide-stabilising tryptophan in the cap domain or halide-stabilising asparagine in the main domain)[2,26,46,47,51,54–56] see Figure 1B.

3. Methods for structural analysis of HLDs

The following section is focused on biophysical methods frequently used to analyze HLDs structure and assess structural changes caused by internal or external factors. The particular attention is paid to circular dichroism (CD) spectroscopy and X-ray crystallography because these two techniques were introduced to Loschmidt Laboratories by the author of habilitation thesis, and simultaneously, they represent the methods of choice for determination of correct folding, stability and atomic resolution structure of HLDs. Below are given information about principles of both methods, attainable types of structural information, and practical examples of the techniques applications on structural analysis of HLDs.

3.1. Circular dichroism spectroscopy

3.1.1. Principle of CD spectroscopy

Plane polarised light is made up of two circularly polarised components of equal magnitude, one rotating counter-clockwise (left-handed, L) and the other clockwise (right handed, R). Circular dichroism refers to differential absorption of these two components. If, after passage through a sample, the L and R components are not absorbed or are absorbed to equal extents, the recombination of L and R would regenerate radiation polarised in original plane (Figure 2). However, if L and R are absorbed to different extents, the resulting radiation would be said to possess elliptical polarisation (Figure 2). A CD signal will be observed when a chromophore is chiral (optically active) for one of the following reasons: (i) it is intrinsically chiral because of its structure, for example, a carbon atom with four different substituents, (ii) it is covalently linked to a chiral centre in the molecule, or (iii) it is placed in asymmetrical environment[57–59]. CD signal arises only when absorption of radiation occurs, and thus spectral bands are easily assigned to distinct structural features of a molecule.

3.1.2. Information available from CD spectra of proteins

An advantage of the CD technique in studies of proteins is that complementary structural information can be obtained from a number of spectral regions. In the far-UV region (typically 250 nm to 190 nm), the principal absorbing group is the peptide bond. The shape (waveform) of CD spectrum is related to geometry (effectively φ/ψ angles) of the polypeptide backbone. Thus, each type of regular secondary structure has a unique spectrum. The spectra in near-UV spectral region (260-320 nm) arise from the aromatic amino acids and disulfide bonds. The actual shape and magnitude of the near-UV CD spectrum of a protein will depend on a number of each type of aromatic amino acid present, their mobility, the nature of their environment (H-bonding, polar groups and polarizability) and their spatial disposition in the protein[58]. The near-UV-visible region (300-700 nm) is useful in proteins with prosthetic groups (e.g., heme proteins, flavoproteins, flavocytochromes, iron–sulfur proteins) because it reflects the conformation and environment of these extrinsic chromophores. Finally, induced CD

signals can arise from ligands which have no intrinsic chirality but acquire chirality when bound in an asymmetric environment such as provided by a protein. The types of information which can be obtained from CD studies of proteins include: (i) secondary structure content of proteins, (ii) tertiary structure fingerprint, (iii) conformational changes in proteins, (iv) protein folding, and (v) ligand binding[58,59]. The examples of structural information most frequently derived from CD experiments on proteins are described below in more details together with particular examples of structural analysis of HLDs by CD spectroscopy.

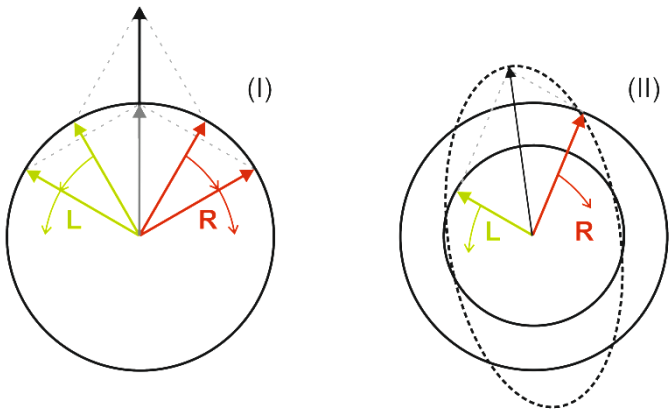


Figure 2. Origin of the CD effect. The left and right (R) circularly polarised components of plane polarised radiation: (I) the both components have the same amplitude and the same phase, and thus, when combined generate plane polarised radiation; (II) the components are of different magnitude and different phase and the resultant (dashed line) is elliptically polarised.

Secondary structure content of proteins

As outlined, the different types of regular secondary structures in proteins give rise to distinct types of CD spectra measured in far-UV wavelength regions. A number of methods have been developed for deconvolution of CD spectra into the calculated secondary structure components present in the protein. These methods include, for example, linear and non-linear least-squares[60], parameterized fit[61], singular-value decomposition[62], linear combination (CONTIN)[63], variable selection (VARSLC, CDSSTR)[64,65], neural network (K_2D, SOMCD)[66,67], and self-consistent methods (SELCON)[68,69]. Many of these methods can be found in worldwide used Dicroprot[70] and DICHROWEB[71–73] packages designed to aid in the determination of secondary structure content. The method of CD spectroscopy has been used for analysis of correct folding and secondary structure of a number of wild-type HLDs during their biochemical and biophysical characterization. Using this technique the correct folding has been confirmed for DbjA from *Bradyrhizobium japonicum* USDA110[38], DmbA and DmbB from *Mycobacterium bovis* 5033/66[37], DmbC from *M. bovis* 5033/66[31], DrbA from *Rhodopirellula baltica* SH1[31], DatA from *Agrobacterium tumefaciens* C58[40], DpcA from *Psychrobacter cryohalolentis* K5[41], DspA from *Strongylocentrotus purpuratus*[19] and DbeA from *Bradyrhizobium elkanii* USDA94[39] (see publications **P3**, **P4**, **P7**, **P11**, **P16**, **P26** and **P32**). Moreover, CD analysis revealed higher α-helical content of DbjA[38] compared to other HLDs due to an 11-amino acid insertion between the main and the cap domain, even before the crystal structure for this enzyme was solved (publication **P3**).

Tertiary structure fingerprint

Although the theoretical treatment of near UV CD spectra is not sufficiently advanced to yield significant structural insights, the near UV CD spectrum of a protein provides a valuable fingerprint of the tertiary structure of the protein. This information can be used, for example, to compare wild type and mutant forms of proteins or to compare the same protein analyzed under different environmental conditions. It should be noted that small conformational changes are more likely to be detected in the near UV than in the far UV region since the CD contributions of the aromatic side chains are usually very sensitive to their environment. This approach was used to assess DbjA conformational stability under different pH conditions[74] (publication **P15**). The near UV CD spectra revealed that tertiary structure of DbjA remain intact within the pH range 6.2–10.1. Comparison of near UV and far UV CD spectra of DbjA determined at various pH conditions unveiled similar pH regions at which the enzyme is structurally stable. Its secondary structure was not affected by pH in the range 5.3–10.3 indicating high robustness of the enzyme[74].

Conformational changes in proteins

CD spectroscopy is very valuable technique for detecting conformational changes in proteins, as the spectra are sensitive even to small alterations in polypeptide backbone structures. This method can be used for comparing the structures of a protein obtained from different sources (e.g. species or expression systems), as was demonstrated on the example of dehalogenase LinB cultivated in *Escherichia coli* and *Pichia pastoris* (publication **P5**). No significant difference between the far-UV CD spectra of LinB expressed in various hosts was found, suggesting that extracellular expression of LinB in eukaryotic system has no effect on the protein fold[75]. CD spectroscopy has been also utilized to compare structures of different mutants of the same protein. This approach has been employed in the following HLD projects: modification of LinB entrance tunnel by saturation of the surface residue L177 (publication **P1**) where CD spectroscopy identified two (out of nineteen) incorrectly folded variants[76]; engineering of DhaA activity towards anthropogenic compound (publications **P8** and **P9**) where all constructed variants were properly folded[12]; substitution of unique halide-stabilizing residue of DatA (publication **P21**) where replacement of the tyrosine with tryptophan had no effect on the overall secondary structure of the enzyme[77]; engineering of DhaA thermal stability and resistance towards organic co-solvent (publication **P18**) where modification of the main tunnel by introduction of bulky and hydrophobic residues did not affect enzyme fold[78]; engineering of DhaA enantioselectivity where insertion of unique surface loop from DbjA into DhaA negatively affect the enzyme secondary structure, but the correct folding was reconstituted by inserting single-point substitutions into its active site and the access tunnel (publication **P31**); engineering of the second halide-binding site in DbeA (publication **P32**) where replacement of the second binding site did not induce conformational changes of the enzyme[39]; or balancing activity-stability trade-off of DhaA variant (publication **P33**) where introduced mutation did not affect the enzyme secondary structure[79]. Furthermore, CD spectroscopy has found utility in studies examining the conformational stability of HLDs under different environmental conditions, such as effects of temperature[1,19,31,37,39–41,74,75,77–83], pH[74], solvents[78–81,84] and ionic strength[39,85] (publications **P4**, **P5**, **P7**, **P11**, **P15-18**, **P20**, **P21**, **P26**, **P30-33** and **P35**). Particularly, thermal denaturation measurements by CD are commonly used to assess the stability of HLDs and are very useful in assessing the effects of amino acid mutations on protein structure[39,77–79,82,83] (publications **P18**, **P21**, **P31-33** and **P35**).

3.2. X-ray crystallography

3.2.1. Principle of X-ray crystallography

The principle of X-ray crystallography is based on X-ray diffraction from crystalline solids that occur as a result of the interaction of X-rays with the electron charge distribution in the crystal lattice. The way X-rays are scattered when they strike the electrons of atoms is similar to the way light waves are scattered by the engraved lines of a diffraction grating. The regular lattice of a crystal acts like a three-dimensional diffraction grating in scattering a monochromatic beam of X-rays, giving a pattern in which the diffracted rays reinforce and do not destructively interfere, as defined by Bragg's equation (Equation 1). According to the Bragg's law, reinforcement, or constructive interference of the monochromatic X-ray beam can only occur if the path difference between rays reflected from successive planes is an even number of wavelengths[86–88] (Figure 3).

$$n\lambda = 2d\sin\theta \tag{1}$$

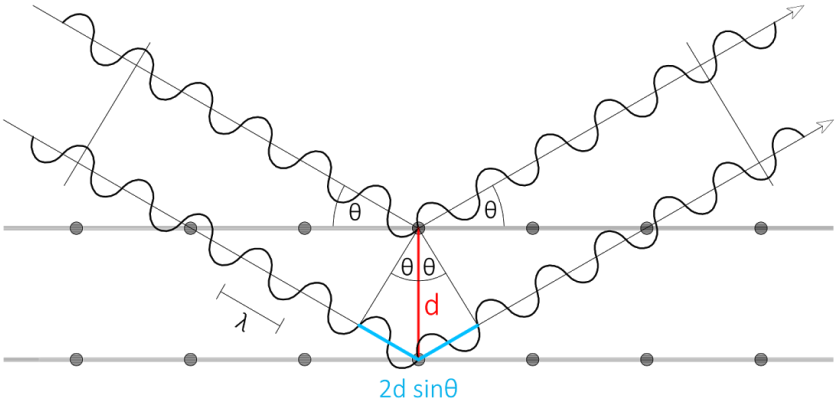


Figure 3. Interpretation of X-ray diffraction as reflection on a lattice plane. The graphical interpretation of the Bragg equation allows treatment of X-ray diffraction as reflection on a set of planes in the crystal. The total path difference between the two excited waves must be equal a multiple of $n\lambda$ for maximum constructive interference. The Braggs equation states this fundamental diffraction condition as $n\lambda = 2d\sin\theta$.

The diffraction process produces X-ray diffraction pattern, which is unique for any given crystal. The structure of the crystal, or more precisely the distribution of its electron density, may be calculated from the diffraction pattern by Fourier transformation. This requires knowledge of the intensities and directions of the diffracted rays and information about their phases. Determination of the phase of each diffracted ray is the most difficult problem. There are two commonly used methods for phase determination in macromolecular crystallography: isomorphous replacement (IR) and molecular replacement (MR)[88–90]. In IR, a heavy metal atom is bound at specific site in the crystal without disturbing its structure or packing. Heavy atom derivatives can be prepared by soaking pre-grown native crystals in heavy atom solutions and/or by co-crystallization of the proteins and heavy atoms together from solution. The heavy atom scatters X-rays more than do the atoms of the protein and its scattering is added to every diffracted ray. Information about the phases of the diffracted rays of the protein can then be obtained from the changes in intensity, depending on whether they are reinforced or diminished by the scattering from the heavy atom[87–89].

MR is a phasing method that uses prior information in the form of known structures that are related or homologous to components in the crystal. Because it requires no additional experimental procedures or data, and in addition simplifies model-building, MR is usually the method of choice for structure determination when a suitable search model is available. The main restriction on the use of MR is the requirement for a suitably similar search model. Good models have low root-mean-square deviation (RMSD) from the target structure and high completeness. Generally, low RMSD between two structures is indicated by high sequence identity. Structures of suitable models are therefore identified by sequence-comparison searches[87–90]. In most cases of successful MR, the protein of interest shares at least 35% sequence identity with its structural homologue, which corresponds to C_α RMSD of around 1.5 Å[90].

Once the phase and amplitude of every diffracted ray are known, the electron density of the protein may be calculated. The structure of protein is obtained by matching this density to the amino acid residues of primary structure using a computer display system. The structure may then be refined by computer methods[87,88,91]. Refinement refers to an iterative process, in which the molecular model is continually fitted and compared against the experimental dataset allowing very slight changes of movement (positional coordinates) and atomic displacement parameters. The changes enable a better agreement between the calculated structure factors (structural model) and observed structure factors (experimental data). The refinement of crystal structure is based on a statistical method, which can provide the crystallographer with a numerical measure of how well the model fits with the experimental data. This statistical measure is known as the least squares method. The "end point" to the refinement process is known as convergence. This is when the structure model is found to be in its best fit to the experimental data. Convergence is said to occur when the overall energy minimum of the structure is found[87,88,92].

3.2.2. Information available from X-ray structures of proteins

X-ray crystallography is the method of choice for obtaining the molecular structure of proteins at atomic resolution. X-ray crystallography can provide very detailed atomic information, showing every non-hydrogen atom in a protein along with atomic details of ligands, inhibitors, ions, and other molecules that are incorporated into the crystal. By using of X-ray crystallography, it is thus possible to investigate how proteins interact with other molecules, how they undergo conformational changes, and how enzymes perform catalysis. In fact, crystallography provides a static model of a dynamic molecule; therefore this model represents an average, in space and time, of three-dimensional (3D) structure of the molecule. However, even the static crystal structure contains useful information for the dynamic nature of the molecule. In the context of protein structures, the B-factors can be taken as indicating the relative vibrational motion of different parts of the structure allowing identification the most flexible parts. Sometimes there are residues in a structure that present clear electron density for two positions, or even multiple distinct conformations. This is direct evidence that the side chains spend time in more than one conformation and thus present a relative protein flexibility[88,93,91].

Important prerequisites for successful protein crystallographic study are (i) preparation of a protein in sufficient amount and purity, and (ii) optimization of conditions to produce diffraction-quality single crystals. Successful crystallization and initial X-ray diffraction analyses of several HLDs and their variants are among others described in publications **P6**, **P12-14**, **P19** and **P24**. The current list of unique tertiary structures of wild-type HLDs solved by X-ray crystallography comprises: DhlA isolated from *Xanthobacter autotrophicus* GJ10[94], DhaA from *Rhodococcus rhodochrous* NCIMB 13064[55], LinB from *Sphingobium japonicum* UT26[46], LinB from *Sphingobium* sp. MI1205[95], DmbA from *Mycobacterium tuberculosis* H37Rv[96], DbjA[9] (publication **P10**), DppA from *Plesiocystis pacifica* SIR-1[32], DmmA from the metagenomic DNA of a marine microbial consortium[33], DatA[97],

DbeA[39] (publication **P32**), HanR from *Rhodobacteraceae* bacterium UDC319[21], DmrA from *Mycobacterium rhodesiae* JS60[22] and DccA from *Caulobacter crescentus*[23]. Besides, more than twenty crystal structures of HLD variants, including variants of DhlA[98,99], DhaA[78,79,82,100,101], LinB[95], DbjA[9] and DatA[97], are available in the Protein Data Bank (see publications **P10**, **P18**, **P29**, **P31** and **P33**).

X-ray analysis of novel or engineered HLDs has uncovered several interesting structural features closely linked to their functionality. For example, crystallographic analysis of DbeA revealed the presence of two fully occupied chloride-binding sites buried in the protein interior[39] (publication **P32**). The first halide-binding site located in the enzyme active site is common to all known HLDs, whereas the second halide-binding site was observed for the first time in the HLD family. The observed spatial proximity of the second halide-binding site to the active-site cavity of DbeA suggests that it may play an important role in the enzyme functionality[39].

X-ray crystallography was employed as well for elucidation of catalytic mechanism of HLDs using DhlA as a model system because at this time it was the only HLD with solved crystal structure. DhlA crystals were soaked into mother liquid containing substrate 1,2-dichloroethane at different pHs and temperatures. These experiments allowed obtaining 3D structures of (i) DhlA with substrate bound in its active site, (ii) DhlA with the covalently bound intermediate and (iii) DhlA with chloride ion as one of the reaction product bound in the active site. The results uncovered that dehalogenation reaction catalyzed by DhlA proceeds by catalytic mechanism with a covalently bound intermediate[54,56]. Since that time, more than 55 structures of enzyme-ligand complexes, mostly complexes of HLDs with the reaction products, have been solved by X-ray crystallography and deposited in the Protein Data Bank. Majority of HLD structures possess the halide anion bound inside the enzyme active site[9,21–23,33,39,46,55,56,78,79,82,84,95,96,100–108]. Several structures of HLDs complexed with alcohol products[21,96,105,107,108] and limited number of HLDs with substrates bound in their active sites[56,96,101,107] are available. The last-mentioned include complexes of DhlA with 1,2-dichloroethane[56], LinB with 1,2-dichloroenthane and 1,2-dichloropropane[107], DmbA with 1,2-dichloroethane[96] and DhaA31 with 1,2,3-trichloropropane[101] (publication **P29**).

Unfortunately, only limited amount of information about solvation and flexibility of HLDs can be directly derived from the crystal structures. Molecular dynamics analyses of HLD structures combined with time-resolved or steady-state florescence spectroscopy were therefore used for site-specific analysis of the enzyme hydration and flexibility. In order to use these spectroscopic methods, selective labelling of the tunnel mouth by various fluorescent dyes or in vivo incorporation of fluorescent unnatural amino acid was necessary[82,109,110] (publications **P25**, **P31** and **P34**). This approach was used for comparison of solvation and flexibility of the tunnel mouths of DbjA, DhaA, and a mutant DhaA12 possessing transplanted active site and the tunnel from DbjA to transfer its catalytic properties to DhaA[82]. Analysis of the time-dependent fluorescence shift together with molecular dynamics simulations revealed that the tunnel mouth of DbjA was the most flexible and hydrated, followed by DhaA12 and DhaA (publication **P31**). Observed differences in hydration and flexibility of tunnel mouths between DbjA and DhaA were further confirmed by time-dependent fluorescence shift experiments performed with the enzymes labelled by various fluorescent probes[109], as well as by steady-state florescence experiments with the enzymes carrying unnatural florescent amino acid introduced into their tunnel mouths[110] (publications **P34**).

4. Function of HLDs

4.1. Catalytic Mechanism

The overall catalytic mechanism of HLDs became apparent when the structure of the first characterized dehalogenase enzyme, DhlA[27], was solved by X-ray crystallography[54,56]. Determination of X-ray structures of the reaction intermediates combined with site-directed mutagenesis[54,56,111] revealed that the catalytic cycle of HLDs proceeds via four main steps: (i) substrate binding, (ii) nucleophilic attack of a carboxylate oxygen of aspartic acid on carbon atom of a substrate to which the halogen is bound, resulting in the formation of a halide anion and an alkyl-enzyme intermediate, (iii) nucleophilic addition of a water molecule, that is activated by catalytic histidine to the ester intermediate, and (iv) release of the reaction products (Scheme 1). Stabilization of the halogen atom of the substrate in the activated complex and the halide anion formed during the dehalogenation reaction is provided by two highly conserved halide-stabilizing residues. The role of the second catalytic acid in the reaction mechanism of HLDs is to stabilise the positive charge that develops on histidine during hydrolysis of the ester[56,112–115].

$$\mathrm{E + RX} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{E.RX} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{E\text{-}R.X} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \mathrm{E.X^-.ROH} \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} \mathrm{E + X^- + ROH} \qquad \text{SCHEME I}$$

4.2. Substrate specificity

HLDs are enzymes with broad substrate-specificity. Individual members of the HLD family are able to convert a wide spectrum of chlorinated, brominated and iodinated alkanes, alcohols, amides, ethers and esters[116]. Systematic exploration of the substrate specificity using a uniform set of halogenated substrates led to the classification of the enzymes into four distinct substrate-specificity groups (SSGs)[117]. HLDs clustered to SSG-I are active towards most of the tested substrates including poorly degradable chlorinated compounds, such as 1,2-dichloroethane, 1,2-dichloropropane, 1,2,3-trichloropropane or 1-chlorohexane. HLDs that predominantly exhibit activity towards terminally substituted brominated or iodinated propanes and butanes are classified as SSG-IV. SSG-II and SSG-III are each represented by one enzyme. SGS-II enzyme exhibits good activity towards 2-iodobutane and *bis*(2-chloroethyl) ether; SGS-III enzyme shows unique preference towards 1-chlorobutane. The study also identified a set of "universal" substrates for analysed HLDs[117], i.e. 1-bromobutane, 1-iodopropane, 1-iodobutane, 1,2-dibromoethane and 4-bromobutanenitrile and the "poor" substrates, i.e. 1,2-dichloropropane, 1,2,3-trichloropropane, chlorocyclohexane and (bromomethyl)cyclohexane. The investigation of the relationship between the function and evolution of HLDs showed that it is not possible to predict the substrate specificity of putative HLDs on the basis of sequence similarities with experimentally characterized family members[117].

4.3. Enantioselectivity

Initial testing of two HLD enzymes, DhlA and DhaA, for their ability to perform kinetic resolution of racemic short chain dihaloalkanes, secondary halogenated alkanes and terminally halogenated esters revealed only a low level of enantioselectivity (*E*-values of up to 9)[118,119]. In contrast, a subsequent investigation of enantiomeric discrimination of several α-brominated esters by DhaA, LinB, and DbjA uncovered a high magnitude of the chiral recognition (*E*-value > 200) for most of the substrates tested[9]. Only DbjA exhibits high enantioselectivity also towards selected β-brominated alkanes (*E*-values of up to 145)[9]. DbjA, LinB, DhaA and its variant DhaA31 were further tested for their ability to

perform kinetic resolution of racemic α-bromoamides. All of the tested enzymes exhibited high enantioselectivity (*E*-value > 200) towards at least one of the tested amides[10]. High enantioselectivity towards α-brominated esters (*E*-value > 200) has been recently identified also for DatA[40], DpcA[41], DbeA and DmxA (unpublished data). DatA[40], DbeA and DmxA (unpublished data) also exhibits high enantioselectivity towards selected β-brominated alkanes (*E*-value ≥ 100). All enantioselective HLDs tested so far have exhibited the preference for (*R*)-brominated substrates[1].

5. Methods for functional analysis of HLDs

Two fundamental questions underscore studies in enzymology and can be addressed by proper kinetic analysis: (i) elucidation of the enzymatic reaction pathway to identify reaction intermediates and to specify the steps that limit the rate of turnover and (ii) quantitative evaluation of the use of binding energy for enzyme specificity and catalysis. In this part of the thesis, kinetic methods used for elucidation of enzyme function are introduced. The general overview starts with steady-state kinetics, followed by the introduction of isothermal titration calorimetry, which is a frequently used method for determination of steady-state kinetic parameters of HLDs. At the end of this chapter are described the methods of transient-state kinetics. Practical examples of kinetic analysis of HLDs are also discussed.

5.1. Steady-state kinetics

The kinetic analysis of an enzyme mechanism often begins by analysis in the steady-state. The steady-state concept generally refers to the situation in which the value of particular quantity is constant – in a *steady-state* – because the rate of formation is balanced by the rate of depletion. In enzyme kinetics, the concept is applied to the concentrations of enzyme-bound intermediates[91]. In steady-state kinetics, initial rates of reactions are measured in a regime in which each enzyme molecule binds substrate and catalyses its conversion to product multiple times. Hence, steady-state kinetics is also frequently referred to as multiple-turnover kinetics[120]. In steady-state kinetic experiments, it is assumed that (i) enzyme (E) and substrate (S) react rapidly to form an enzyme substrate complex (ES) which breaks down directly to form free E and product (P) (Scheme 2). (ii) Only a single S and a single ES are involved in the reaction. (iii) E, S, and ES are at equilibrium. (iv) The overall rate of the reaction is limited by the breakdown of ES to form the free E and P[91,121,122].

$$\mathrm{E + S} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{ES} \xrightarrow{k_2} \mathrm{E + P} \qquad \text{SCHEME II}$$

The experiments must be set up such that substrate concentration is very much higher than the enzyme concentration so that the formation of an ES does not alter the substrate concentration. It is also important to measure the rates of the reactions during the early stages so that the reverse reaction is insignificant. To perform a steady-state kinetics experiment, the initial rates of the reaction at a fixed enzyme concentration are measured as a function of substrate concentrations (Figure 4). Under these conditions, the rate of the reaction is given by the Michaelis–Menten equation:

$$v = \frac{[\mathrm{S}][\mathrm{E}]_0 k_{\mathrm{cat}}}{K_{\mathrm{m}} + [\mathrm{S}]} \qquad (2)$$

The quantity $[\mathrm{E}]_0 k_{\mathrm{cat}}$ is called the V_{lim}, the maximal rate of the reaction at a given concentration of enzyme. V_{lim} is the rate of enzyme-catalyzed reaction approaches at very high substrate concentrations (i.e., saturation). k_{cat} is the apparent first-order rate constant for the enzyme-catalyzed reaction at

saturating concentrations of substrate. It reflects the slowest step (or steps) along the reaction pathway after formation of the enzyme substrate complex. k_{cat} is often called the *turnover number* of the enzyme because it represents the maximum number of substrate molecules converted to products per active site per unit time. K_m, the Michaelis constant, is the concentration of the substrate at which $v = V_{lim}/2$. In steady-state concept, K_m is considered as apparent dissociation constant of ES complex and is a reflection of enzyme affinity towards individual substrate. The ratio k_{cat}/K_m, often referred to as the specificity constant, defines the apparent second-order rate constant for the reaction between substrate and free enzyme. This ratio allows direct comparison of the effectiveness of an enzyme towards different substrates[91,122,123].

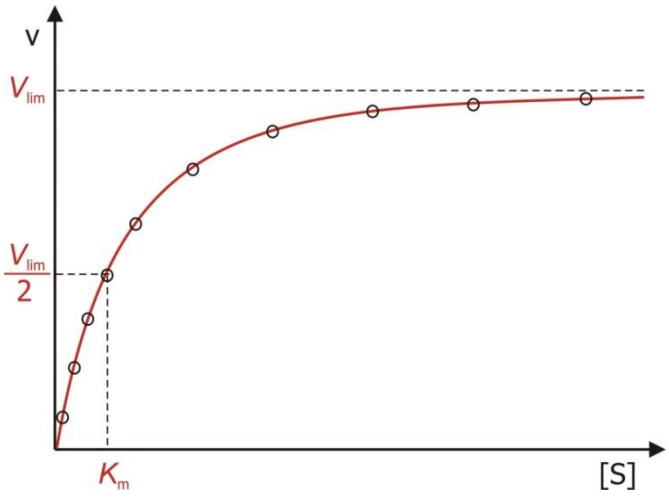


Figure 4. Reaction rate of enzyme plotted against substrate concentration for reaction obeying Michaelis-Menten (or saturation) kinetics.

Steady-state kinetic data can be collected either by discontinuous or continuous methods. A wide variety of assays can be used to perform steady-state enzyme kinetics experiments. These usually include radioactivity-, absorbance-, and fluorescence-based assays[122]. Since virtually all enzymatic reactions proceed with the exchange of heat with the environment, calorimetric methods can be employed as well to monitor the progress of enzyme-catalyzed reactions[124]. Calorimetric methods, particularly isothermal titration calorimetry (ITC)[124–127], have many advantages compared to other methods. These advantages include no need of any specific spectroscopic requirement, no chemical derivatization to facilitate spectroscopic measurements, no coupling reaction and no other analytical method to determine product concentration. ITC experiments are conducted as continuous assays; they are rapid, simple, precise, non-destructive and sensitive. Since ITC represents a frequently used method to determine steady-state kinetic parameters of HLDs, basic introduction of the method is given bellow.

5.1.1. ITC technique

ITC can be used to measure enzyme kinetic parameters because the thermal power generated as the reaction proceeds is a direct and sensitive observable event[124–127]. ITC calorimeter consists of two cells (sample and reference) maintained at constant temperature through the continuous addition of thermal power (Figure 5). The temperature difference between the reference cell and the sample cell is measured, and a variable level of thermal power is supplied to the sample cell in order to drive this temperature difference to zero. The thermal power supplied to the sample cell represents the raw

signal. Enzyme reaction rates are determined by measuring the change in instrumental thermal power supplied to the sample cell after addition of the substrate or enzyme through a stirred injection syringe.

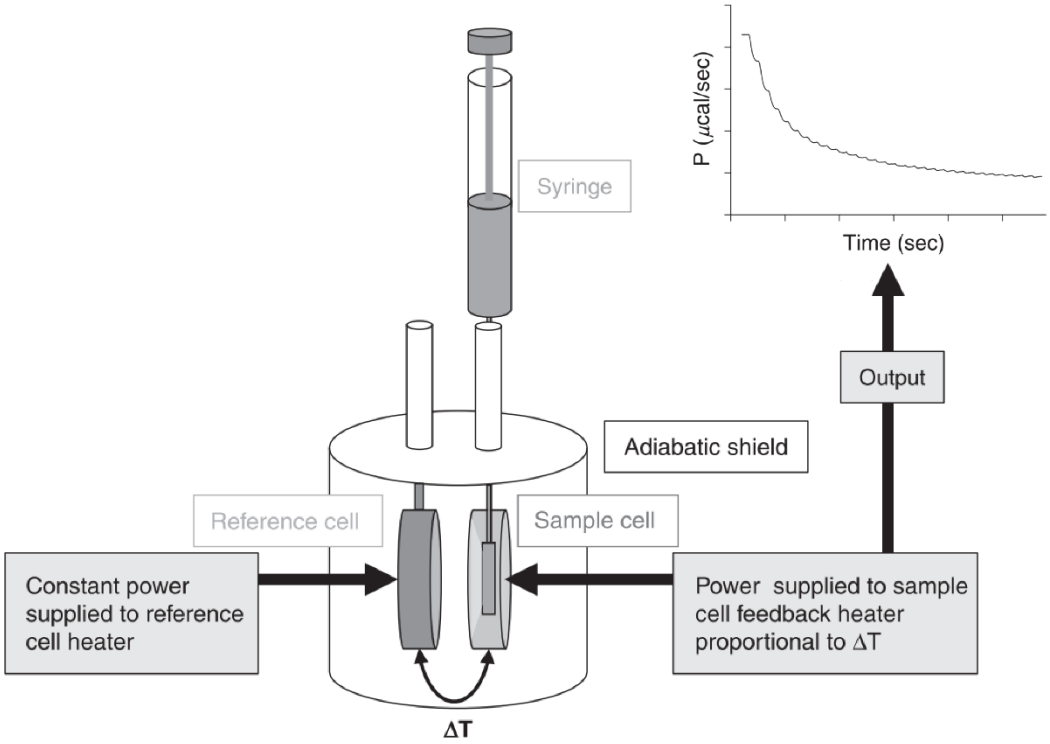


Figure 5. Representative diagram of a typical ITC. Main components of the instrument, the reference and sample cells, syringe for adding titrant, and the adiabatic shield, are depicted in the figure. This diagram shows an oversimplification of how the power applied by the instrument to maintain constant temperature between the reference and sample cells is measured providing the output signal. Redrawn from the publication of Freyer et al.[126].

Heat (Q) measured as a function of time (dt) is defined as the thermal power:

$$\text{Pover} = \frac{dQ}{dt} \tag{3}$$

The amount of heat associated with converting n moles of substrate to product is given by:

$$Q = n \cdot \Delta H_{\text{app}} = [P]_{\text{Total}} \cdot V \cdot \Delta H_{\text{app}} \tag{4}$$

where V is the volume of the solution in the reaction cell, P is the molar concentration of product generated, and ΔH_{app} is an experimentally determined molar enthalpy for the reaction. Measured thermal power generated by an enzyme is thus proportional to the reaction rate:

$$\text{Pover} = \frac{dQ}{dt} = \frac{d[P]}{dt} \cdot V \cdot \Delta H_{\text{app}} \tag{5}$$

Providing, after rearrangement:

$$\text{Rate} = \frac{d[P]}{dt} = \frac{1}{V \cdot \Delta H_{\text{app}}} \cdot \frac{dQ}{dt} \quad (6)$$

Reaction rates (in units of power) can be converted to enzyme turnover if the apparent enthalpy (ΔH_{app}) is known. The ΔH_{app} can be determined by allowing the reaction to proceed to completion, and then integrating the signal to obtain the total heat evolved[125]:

$$\Delta H_{\text{app}} = \frac{1}{[S]_{\text{Total}} \cdot V} \int_{t=0}^{t=\infty} \frac{dQ(t)}{dt} \cdot dt \quad (7)$$

There are two different ITC methods for performing an enzyme kinetic experiment: (i) single injection and (ii) multiple injections experiments. When performing a single injection ITC kinetic experiment, the enzyme solution is injected into the cell containing the substrate solution producing a heat response which eventually returns to baseline after all of the substrate has reacted. The multiple injections of ITC kinetic experiment makes use of multiple titrations at intervals that are spaced such that subsequent titrant additions are performed when the heat rate has reached a steady state. It is important that each subsequent addition of substrate is made prior to significant reaction of the substrate. The difference in the signal plateau between each injection is used to determine the reaction rate at that step. This information is used to create a plot of the reaction rate (in units of power) versus total substrate concentration. The main advantage of the multiple injections method over the single injection method is higher accuracy in determining kinetic parameters. The only disadvantage is that it is unable to determine ΔH_{app} because the titration points do not allow for the complete reaction of the substrate prior to adding more substrate. This can be overcome by performing a single injection experiment to determine the ΔH_{app}[126].

Several kinetic experiments employing ITC have been performed with HLDs. Steady-state kinetic assays with ITC were conducted to determine how selected organic co-solvents affect kinetic behaviour of DbjA, DhaA and LinB with 1-iodohexane as a substrate[84] (publication **P22**). Complex kinetic mechanism including cooperativity and substrate inhibition was uncovered by ITC for all studied enzymes in the absence of co-solvents. Addition of tested co-solvents either enhanced catalysis by lowering $K_{0.5}$ and increasing k_{cat}, or caused enzyme inactivation by promoting substrate inhibition and decreasing k_{cat}[84].

Conversion of 1-chlorohexane by LinB and its variants LinB D147N and LinB L177D in the presence and the absence of various chloride salts was monitored by ITC to investigate dependence of the enzyme catalytic efficiency on the binding of cations on the protein surface, particularly at the tunnel mouth leading to the enzyme active site[85] (publication **P23**). ITC revealed that kinetics of LinB and its variants with 1-iodohexane did not follow a simple Michaelis-Menten dependence. Instead, the velocity data were fitted by a more complex kinetic scheme, including complexes of the enzyme with two or three substrate molecules. The population of unproductive ESSS complexes was enhanced when D147N mutation was introduced at the tunnel mouth of LinB, while the mutation L177D reduced the substrate binding to the ESS complex. Both formation and productivity of ESSS complexes were inhibited more strongly by Na^+ and K^+ than Rb^+ and Cs^+ ions[85].

The method of ITC was further used for determining the steady-state kinetic parameters of DbeA possessing two halide-binding sites and its variant lacking the second halide-binding site (DbeAΔCl)[39] (publication **P32**). DbeA kinetics with 1-bromobutane followed a mechanism involving cooperativity and substrate inhibition. Importantly, DbeAΔCl exhibited 100 times lower k_{cat} as well as decreased K_{m} compared to the wild-type enzyme, suggesting that elimination of the second halide-binding site

significantly reduced catalytic activity of the enzyme. Moreover, substrate inhibition was not observed with DbeAΔCl variant[39].

5.2. Pre-steady state kinetics

In order to determine the rate constants of the individual steps on the reaction pathway and detect the transient intermediates, it is necessary to measure the rate before the steady-state phase is reached. It is during the time period in which the steady-state is set up that the individual rate constants of arising and decaying transient enzyme-bound species may be observed. Since value of k_{cat} lies between 1 to 10^7 s^{-1}, measurement must be made in a time range of 1 to 10^{-7} s. This requires special techniques for measurement[91].

5.2.1. Stopped-flow and rapid quench-flow methods

Transient-state kinetic analysis is most commonly based upon stopped-flow methods where an optical signal is used to follow the time dependence of a reaction; however, it is often difficult or impossible rigorously interpret the optical signal. For example, if the absolute extinction coefficients and concentrations of species contributing to the optical signal are not known, then the reaction pathway cannot be determined unambiguously. These problems are solved by use of chemical-quench-flow methods, in which the intermediates in a reaction sequence can be quantified directly after the reaction is stopped by the addition of a quenching agent, such as acid, base or other denaturant[128].

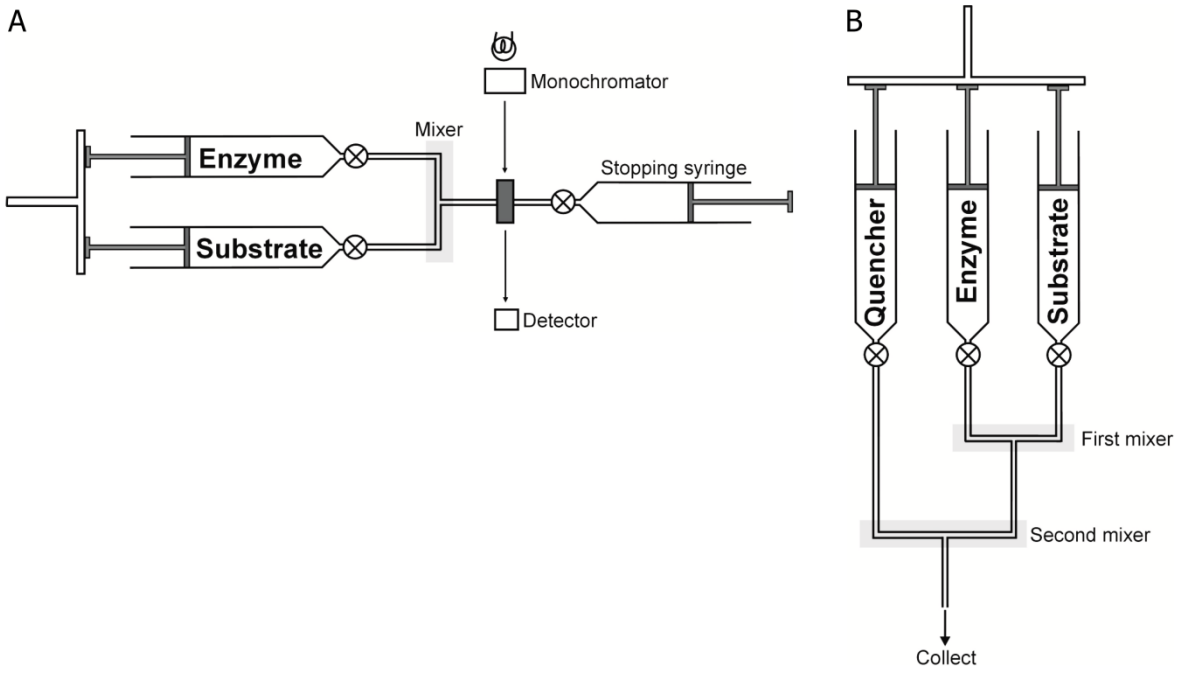


Figure 6. Schematic diagrams of stopped-flow (A) and quenched-flow (B) apparatus.

The stopped-flow apparatus (Figure 6A) allows the rapid mixing of two or more solutions, which then flow into an observation cell while the previous contents are flushed and replaced with freshly mixed reactants. A stop syringe is used to limit the volume of solution expended with each measurement and also serves to abruptly stop the flow. The reaction is followed as the solution ages after the flow stops. The time resolution of these methods is limited by the time required for the reactants to flow from the point of mixing to the point of observation: this "dead time" is typically on the order of 1-4 ms, depending on the apparatus[91,123].

The rapid quenching apparatus (Figure 6B) allows the mixing of reactants, followed, after a special time interval, by mixing with quenching agent, to denaturate the enzyme. The quenched sample is then collected and analysed to quantitate the conversion of substrate to product or intermediate, usually by chromatographic methods[123,128,129]. The time of reaction is determined by the volume of the reaction loop between two points of mixing and the rate of flow. In practice, the reaction time is varied by changing the length of tubing in the reaction loop and, to a lesser extent, by changing the rate of flow. By selecting various reaction loops, times from 2 to 150 ms can be obtained[123].

For detailed kinetic measurements, two types of pre-steady-state experiments – a multiple and a single turnover – can be conducted. The main difference between these two experimental conditions is stoichiometry of enzyme states. Multiple turnover experiments are carried out under the conditions where initial concentration of substrate is always in excess over enzyme. When enzyme is mixed with substrates (in excess over the enzyme), a burst of product formation at a rate faster than steady-state turnover can be observed. The occurrence of the burst indicates that a step after chemistry is at least partially rate limiting. If the burst of product formation is not observed, then either the chemical reaction or a step preceding the chemistry is rate limiting. Single turnover experiments, in ideal cases, are performed with enzyme in excess over substrate and thereby examine 100% conversion of substrate to product in a single enzyme turnover with a high degree of sensitivity in looking for enzyme intermediates[120,123,130].

Several kinetic studies focused on identification of the rate-limiting step in the catalytic cycle of various HLDs have been conducted to date. The pre-steady-state kinetic methods comprising stopped-flow fluorescence and rapid quench flow measurements have been performed with DhlA[112,131], DhaA[113], LinB[114,132], DatA[77], DbeA[39] and their variants[12,39,77,133]. It has been shown that the rate-limiting step in the catalytic cycle is halide release in the case of DhlA reacting with 1,2-dichloroethane and 1,2-dibromoethane (Schanstra et al., 1996), release of an alcohol and cleavage of the carbon–halogen bond for DhaA with 1,3-dibromopropane[113] and 1,2,3-trichloropropane[12] (publication **P8**), respectively, hydrolysis of the alkyl-enzyme intermediate for LinB with 1-chlorohexane, chlorocyclohexane and bromocyclohexane[114] (publication **P2**), and hydrolysis of the alkyl-enzyme intermediate for DatA[77] and DbeA[39] with 1-bromobutane (publications **P21** and **P32**). The observed differences in the rate-limiting step suggest that the catalytic efficiency of HLDs is governed by the particular enzyme–substrate pair and depends on: (i) the composition of the catalytic residues, (ii) the geometry and solvation of the enzyme active-site cavity and (iii) the geometry and dynamics of the access tunnels connecting the buried enzyme active site with the surrounding solvent[2,12,103,114].

6. Engineering of HLDs

Efficient application of enzymes in biocatalysis and biotransformations on an industrial scale requires substantial improvements of wildtype enzymes to make them robust. Typical goals of enzyme modification are focused on improved substrate specificity (narrower or broader substrate scope depending on the application, altered regio- and stereoselectivity, increased reaction rate, elimination of substrate/product inhibition), and better tolerance to reaction conditions (pH, heat, solvents, reactive components, high substrate concentrations in the molar range)[134]. Many complementary strategies were developed to improve enzyme performance. These strategies can be grouped into four general categories: (ii) modification of enzyme structure (protein engineering), (iii) modification of solvent environment (medium engineering), (iii) searching for existing enzymes with unique properties in metagenomic libraries, and (iv) tailor-made enzyme immobilization[135–140] (see publication **P27**). In this part of the thesis, first two strategies are discussed together with selected examples of effective modification of HLD properties.

6.1. Protein engineering

Protein engineering usually follows two major concepts: rational design or directed evolution[134,136]. Rational protein design (Figure 7) requires the 3D-structure of a protein or at least a good homology model. A high-resolution structure of an enzyme is usually determined by X-ray crystallography. A model of the protein structure can be generated automatically based on a known homologous structure or alternatively can be predicted from evolutionary and sequence variations. These structures then serve as a basis to perform molecular modeling for the identification of key residues to be changed in order to improve the protein in the desired direction[134,136,141,142]. Once amino acid residues are identified from a structural analysis these are introduced into the protein on the gene level by site-directed mutagenesis[143] and after production of the mutant it can be analyzed for altered function.

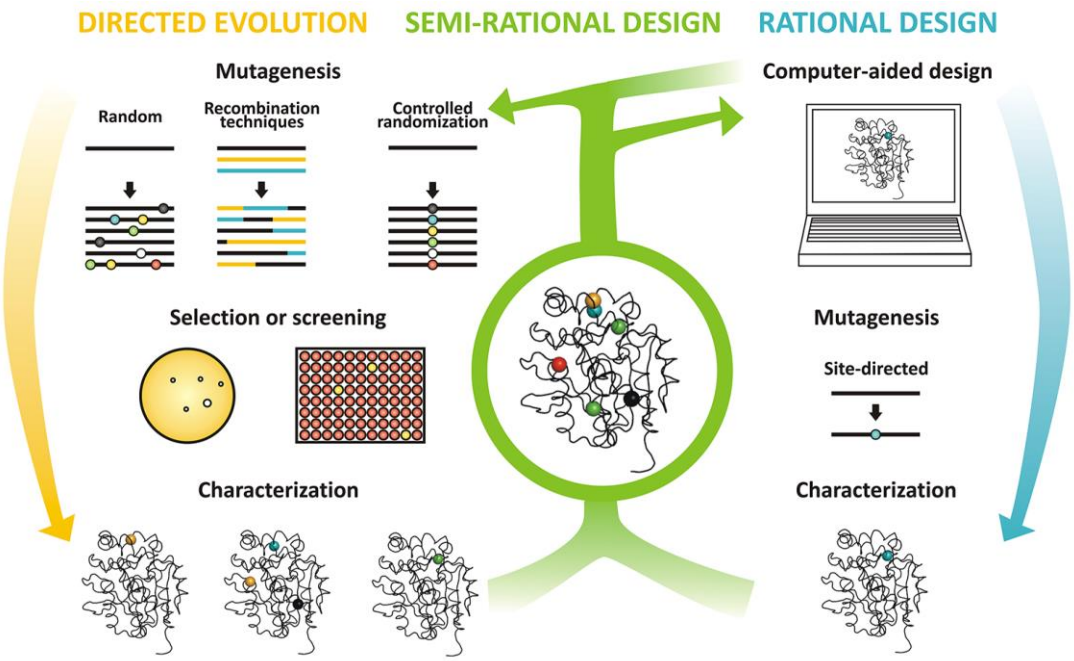


Figure 7. Overview of protein engineering strategies[135].

In contrast, directed evolution (Figure 7) only requires a template DNA for the functional expression of the gene to produce the protein of interest. The protein-encoding gene is then subjected to random mutagenesis (e.g., by error-prone PCR), controlled randomization at specific positions or gene shuffling resulting in very large mutant libraries. These are then either screened for the desired property or subjected to selection methods. Best hits are usually subjected to further rounds of directed evolution until the protein shows the desired properties. Finally, the position and type of amino acid substitutions are determined and the enzyme is biochemically characterized in detail. Back mutations or combinations of selected mutations are often performed to further improve the enzyme or elucidate the specific influence of a given amino acid substitution[134,144,145]. The latest trend is to combine these two approaches and generate small, functionally rich mutant libraries ("small but smart" libraries) using rationally preselected target sites and limited amino acid diversity in a so-called semi-rational design[136,146,147] (Figure 7).

Different approaches of protein engineering have been applied to modify properties of HLDs. Rational design strategy was used to engineer HLDs stability[148,149,83], activity[39,77,150], substrate specificity[151], binding efficiency towards fluorescent probe[152], enantioselectivity[9,82], and to confirm the role of putative catalytic residues in early stages of HLDs characterization[38,47,55,111,153–158]. Directed evolution approach was employed in the engineering of HLDs resistance towards organic co-solvents[78], thermal stability[78,159] and catalytic activity[159–162]. A combination of directed evolution and rational design has been applied to modify HLD activity[12,76], substrate specificity[76], stability towards elevated temperature and organic co-solvents[78] and balance the activity-stability trade-off[79]. The important structural features of HLDs often targeted by mutagenesis to improve their catalytic and process performance include: (i) access tunnels connecting buried enzyme active site with the surrounding solvent and (ii) catalytic pocket residues (except catalytic residues) defining the size and nature of the binding site cavity and participating in substrate binding and product release[12,76,78,79]. Below are listed the most recent examples of protein engineering studies of HLDs which are attached to this thesis.

6.1.1. Engineering stability of HLDs

Eight variants of haloalkane dehalogenase DhaA were prepared by random mutagenesis, site directed mutagenesis and saturation mutagenesis with a goal to improve resistance of the enzyme towards 40 % (v/v) of DMSO. Structural and biochemical characterization of DhaA variants revealed that only the mutations in the access tunnel were essential for the improved structural and kinetic stability, while the variants with mutations on the surface exhibited similar stability parameters as the wild-type enzyme. Four mutations introduced cumulatively to access tunnel of DhaA resulted in correctly folded protein (DhaA80) with 4000-fold improved kinetic stability in 40 % (v/v) DMSO, elevated structural thermostability by 16 °C, but 100-fold lower catalytic activity with 1,2-dibromoethane in pure buffer compared to the wild type enzyme. The stabilization was achieved by enhanced intramolecular hydrophobic packing at the tunnel opening and possibly preventing the destabilisation of the protein structure by admission of DMSO molecules to the active site due to the introduced hydrophobic and bulky residues[78] (publication **P18**).

Follow-up study was aimed to improve catalytic activity of the highly stable DhaA80 in buffer, with minimum loss of its stability (publication **P33**). Systematic mutagenesis of two of the four originally modified tunnel residues (F176 and V172) resulted in a single point variant F176G possessing 32- and 10-times improved catalytic activity in buffer and in 40 % (v/v) DMSO, respectively. Thermostability of the mutant was lowered by 4 °C only. Moreover, the newly evolved variant exhibited enhanced activity towards 26 out of 30 tested halogenated compounds similarly to wild-type enzyme. Structural analysis and molecular dynamics revealed that newly introduced mutation F176G reopened previously

closed tunnel in stable DhaA and increased the mobility of the two α-helices lining the tunnel, thus restoring the enzyme activity, while remaining tunnel mutations maintained its stability[79].

The recently published computational method called FireProt was used to enhance the stability of DhaA (publication **P35**). This approach searches *in-silico* for mutations likely to stabilize a protein of interest via: i) enthalpy calculations of potential point mutants (energy-based mutations) and ii) phylogenetic identification of residues that have drifted from potentially more stable consensus sequences (evolution-based mutations). In FireProt, these calculations are combined using smart filtering to allow the design of highly stabilized multiple-point mutants[83]. The best DhaA variants designed by energy-based approach, evolution-based approach, and by their combination exhibited melting temperature increased by 16, 10 and 25 °C, respectively[83].

6.1.2. Engineering activity of HLDs

In order to improve catalytic properties for TCP conversion, several positions in the access tunnels of DhaA were selected for saturation mutagenesis. This selection was performed based on the results from random acceleration molecular dynamics exploring the pathways and the mechanisms of 2,3-dichloropropane-1-ol and chloride release. The most successful variant, DhaA31, exhibited a 32-fold higher catalytic activity and a 26-fold higher catalytic efficiency for TCP than the wild-type enzyme. This variant had bulky aromatic residues at four of the five targeted positions, which lead to the occluded active site cavity. The rate-limiting step of TCP conversion in the resulting variant was shifted from carbon–halogen bond cleavage to the release of the reaction products[12] (publication **P8**).

6.1.3. Engineering substrate specificity of HLDs

Tunnel-mouth engineering was shown to have profound effects on the activity and specificity of LinB (publication **P1**). The residue L177, located in the tunnel opening was selected for saturation mutagenesis on the basis of structural and phylogenetic analyses. The effects of the resulting mutations on the variants' catalytic activities greatly differed for individual substrates, and thus the mutants exhibited different specificity profiles. The catalytic activity of LinB variants was generally increased by introducing a small non-polar amino acid at position 177, whereas the introduction of bulky aromatic or charged residues dramatically reduced activity towards most tested substrates. The small side chain of the introduced glycine residue in the LinB L177G mutant was proposed to increase the radius of the tunnel mouth, thereby facilitating substrate entry and product release. Conversely, the bulkier side chain of the introduced tryptophan residue in the LinB L177W variant presumably blocked the mouth of the enzyme's main access tunnel, reducing its catalytic activity[76].

Engineering of the unique second halide-binding site identified in the structure of DbeA was discovered as a possible strategy to modify substrate specificity of HLDs (publication **P32**). Construction and biochemical characterization of a two-point mutant of DbeA (I44L+Q102H) lacking the second halide-binding site revealed decreased stability of the enzyme in the presence of chloride salts and decreased catalytic activity by an order of magnitude. Moreover, the two-point substitution resulted in a shift of the substrate-specificity class, which is the first time this has been demonstrated for the HLD enzyme family. Changes in the catalytic activity of the variant were attributed to deceleration of the rate-limiting hydrolytic step, mediated by lower basicity of the catalytic histidine[39].

6.1.4. Engineering enantioselectivity of HLDs

Enantioselectivity of DbjA towards β-brominated alkanes and α-brominated esters was modulated by deletion of the unique surface loop between the core and the cap domain and by a point mutation (His139Ala) located next to the deleted loop (publication **P10**). The loop deletion and the subsequent point substitution decreased and restored enzyme's selectivity towards 2-bromopentane, respectively. On the other hand, the deletion improved the selectivity towards (*R*)-methyl 2-bromobutyrate. The results from mutagenesis together with substrate mapping, X-ray crystallography, thermodynamic analysis and molecular modelling implied two different bases of enzyme's enantioselectivity towards two distinct groups of substrates, β-bromoalkanes and α-bromoesters[9].

Engineering of DhaA enantioselectivity towards β-brominated alkanes by transplanting the active site and access tunnels from closely related DbjA possessing high enantioselectivity to nonselective DhaA failed. Although the transplantation was successful and the active-site geometries of the constructed DhaA variant and DbjA were identical; enantioselectivity of the redesigned enzyme (DhaA12) towards the target substrates remained unchanged. Computer simulations coupled with time-dependent fluorescence shift pointed out different hydration and dynamics of the tunnel mouth between DhaA, DhaA12 and DbjA as the probable cause of the failure. Undoubtedly, dynamics and hydration are important for enzymatic catalysis and should be considered in rational design of HLDs enantioselectivity[82] (publication **P31**).

6.2. Medium engineering

The field of medium engineering was developed based on the observation that the performance and even the specificity of enzymes can be modulated through the solvent where the reaction is made to occur[163]. Although this approach does not directly influence the enzyme's sequence, it produces conformational effects affecting its function. The main factor that has to be taken into account when performing biocatalysis in nonconventional media is water content. It is generally believed that fully dehydrated proteins are inactive[164]. Water is able to participate directly in enzyme-catalyzed reactions as a substrate, and/or during the transitions states and/or as reaction product. Additionally, water acting as a lubricant promotes the conformational mobility required for optimal catalysis. On the other hand, if the water content of an organic solvent exceeds a certain limit, the tendency of an enzyme to denature is increased because of higher conformational mobility[135,137].

Different types of reaction media for enzymatic reactions have been describe and can be classified into the following categories: (i) aqueous solvents, (ii) organic solvents (monophasic organic systems), (iii) water: water-miscible (monophasic aqueous-organic systems), (iv) water: water-immiscible (biphasic aqueous-organic systems), (v) supercritical fluids, (vi) anhydrous media, (vii) reversed micelles, (viii) solvent-free systems, (ix) gas phases, (x) fluorinated solvents, (xi) ionic liquids, and (xii) deep eutectic solvents[137,165–167]. Besides, dissolved salts can be considered as another class of co-solute often found in bioprocessing media, as buffer components or precipitating agents[135,137,168,169]. Addition of inorganic salts to an aqueous enzyme solution could significantly affect its performance, since ion specific effects play an important role in the biochemical and biophysical processes. This part of the thesis describes the scopes and limitations of enzymes in selected non-conventional media, including water miscible organic co-solvents, deep eutectic solvents and inorganic salts. Practical examples of investigation of HLDs performance in these non-conventional media are also presented.

6.2.1. Organic co-solvents

Organic co-solvent systems are produced when water-miscible solvents are added to the aqueous medium to improve the solubility of compounds sparingly soluble in water, to modify the enzyme properties or to decrease the water content in the reaction medium to favour synthesis over hydrolysis[170,171]. Surprisingly, enzymes are much more tolerant to pure organic solvents than to water-solvent mixtures[172]. This is due to the interplay of the two effects. On the one hand, as the organic solvent concentration is raised the tendency of protein to denature is increased. On the other hand, as the water content declines, the protein conformational mobility is diminished. Increase of organic co-solvent concentration in aqueous media generally decreases the enzyme activity[164]. Most enzymes become almost totally inactive at an organic co-solvent concentration of 60-70 % (v/v)[173]. The reported reasons for alterations of enzyme activity by the addition of organic co-solvents are: (i) changes of protein conformation, (ii) competitive inhibition of an enzyme, and (iii) changes of properties of the medium, such as dielectric constant, polarity or hydrophobicity[84,174,175].

Conformational changes are the most common reason for enzyme deactivation in the presence of organic co-solvents. Miscible solvents are known to turn the hydrophobic core of the protein from buried to more exposed state. Solvents having higher log*P* and high solvation capacity, like 1,4-dioxane, tetrahydrofuran or isomers of butanol, are strong denaturants causing inactivation at concentrations as low as 10-30% (v/v), whereas more hydrophilic solvents like glycerol, ethylene glycol or formamide can be often used at concentrations of 50-60% (v/v)[137,176]. On the other hand, polar organic solvents that can penetrate into the protein core are more capable of causing the disruption of the protein molecule than non-polar solvents[170,177].

Comprehensive screening of the effect of various concentrations of 14 water-miscible organic solvents on structure, stability and activity of three HLDs, DbjA, DhaA and LinB, displayed different resistance of individual enzymes towards the solvents[80] (publication **P20**). An increase in DbjA activity was induced by the majority of organic co-solvents tested, while activities of DhaA and LinB decreased at comparable concentrations of the same co-solvent. Circular dichroism and fluorescence spectroscopy revealed that enzyme inactivation was connected with alteration of their structure, however, activity changes were observed also at non-denaturing solvent concentrations. Moreover, a high increase of DbjA enantioselectivity was observed (publication **P20**). Ethylene glycol and 1,4-dioxane were shown to have the most positive impact on the enzyme enantioselectivity[80].

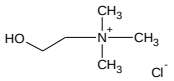
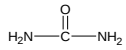
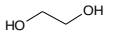
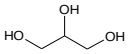
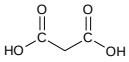
To elucidate the mechanism of solvent-enzyme interaction at the molecular level, steady-state kinetics molecular modeling simulations and time-resolved fluorescence spectroscopy in the presence of three selected solvents - acetone, formamide, and isopropanol - were assessed. It was found out that co-solvent molecules entered the enzymes' access tunnels and active sites, enlarged their volumes with no change in overall protein structure, but surprisingly did not act as competitive inhibitors. Correlation between observed solvent-induced activation/inhibition of enzyme and the portion of the enzyme cavity occupied by solvent molecules during simulation was revealed[84] (publication **P22**).

6.2.2. Deep eutectic solvents

Deep eutectic solvents (DESs) have been described as new class of ionic solvents, obtained by complexation of quaternary ammonium salts and hydrogen bond donors that melt at low temperatures due to the charge delocalisation[166]. At the eutectic ratio, typically 1-4 molecules of hydrogen bond donor per molecule of salt, the mixtures form a liquid at room temperature. DESs were established by Abbott et al.[166], who reported low melting mixture of (2-hydroxyethyl) trimethyl-ammonium (choline) chloride (ChCl), so called vitamin B4, and urea. Consequently, different hydrogen bond donors, such as alcohols, carboxylic acids and urea derivatives, were used in combination with ChCl or

ethylammonium chloride[166,178,179]. Examples of commercially available DESs are listed in Table 1. DESs represent a green alternative to classical organic and ionic solvents, because they are straightforward and inexpensive to prepare, do not require extensive purification, and are expected to be environmentally friendly[81,167,179].

Table 1. Examples of the commercially available DESs.

Component 1		Component 2		DES	Ratio	T_f (°C)
Choline chloride		Urea		Reline	1:2	12
		Ethylene glycol		Ethaline	1:2	-20
		Glycerol		Glyceline	1:2	-40
		Malonic acid		Maline	1:1	10

T_f – freezing point[179].

First utilization of DESs in enzyme-catalysed reactions was described by Gorke et al. who assess the activity of different hydrolytic enzymes in ChCl-urea mixtures[180]. Despite high concentration of urea, which is a strong hydrogen bond donor and denatures protein, and the presence of halides, which may inactivate or inhibit the proteins, the enzymes exhibited good catalytic activity. In particular, the conversion of styrene oxide to the corresponding diol with epoxide hydrolase was 20-fold enhanced using choline chloride – urea as a co-solvent[180]. Since that, positive effect of various DESs used as co-solvents on activity, stability and enantioselectivity of several hydrolytic and oxidoreductase enzymes has been reported[81,167,181–186].

Catalytic performance of three HLDs, DbjA, DhaA and LinB, in aqueous solutions of DES ethaline was systematically and critically compared with the enzyme reactivity in the presence of the individual components of ethaline and two conventional organic solvents, methanol and acetone (publication **P30**). The activities of the studied enzymes responded differently to the different co-solvents. All of the enzymes tolerated ethaline and ethylene glycol significantly better than methanol and acetone. Haloalkane dehalogenase DhaA was found to be the most tolerant of ethaline. Its excellent compatibility with the solvent was demonstrated by the enzyme's enhanced thermal stability, and by its retention of detectable catalytic activity even at very high ethaline concentrations (≥ 90% v/v). In contrast, the activities of DbjA and LinB were higher in ethylene glycol than in ethaline. Moreover, the activity of DbjA was 1.5 times higher in 50% (v/v) ethylene glycol than in pure buffer. Additionally, the enantioselectivity of DbjA increased more than 4-fold in the presence of ethaline or ethylene glycol. The results demonstrated that ethylene glycol and DES ethaline can have beneficial effects on catalysis by HLDs[81].

6.2.3. Inorganic salts

Historically, salts have been classified in a lyotropic series (or Hofmeister series) of ions based on their ability to change water structure by ionic hydration (hydrogen bonds). Kosmotropic ions are able to establish hydrogen bonds; meanwhile chaotropic ions just break them. The same effect on proteins is related to the salting in or salting out phenomena, and subsequently on the stability of secondary and tertiary structures[137]. In opposite sides, ions were classified as protein structure stabilizers or disruptors, as illustrated in Figure 8. Although there has been an intensive effort devoted to explaining ion effects on enzymes at the molecular level through both theoretical and experimental approaches,

an understanding of these effects is still incomplete. Specifically, an ion may affect the enzyme activity by playing the role of a substrate, a cofactor, or an inhibitor. Generally, ions may affect the enzyme activity as well as stability indirectly by altering the bulk water structure, by affecting the enzyme-water interface or directly by binding to the enzyme molecule[187–189]. The indirect effects are stronger for anions than cations due to a greater effect of anions on water ordering[137]. However, also direct interactions of ions with the enzyme surface and the active site was found to be responsible for enzyme activation/deactivation[190,191]. Therefore, cationic effects on enzyme properties also have to be taken into account, especially when the enzyme surface is negatively charged[85].

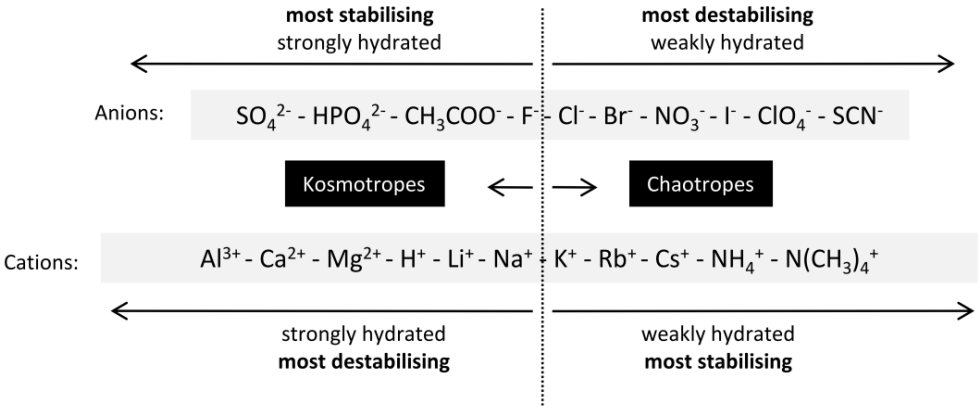


Figure 8. Hofmeister series describing the arrangement of ions according to their ability to modify the properties of proteins, via their effectiveness in altering the internal structure of the bulk water phase. Chaotropes disrupt water structure, while kosmotropes stabilise it.

Interactions of individual alkali cations with HLDs surface and their effects on enzymatic activity were investigated by combination of molecular dynamics simulations and enzyme kinetic experiments (publication **P23**). Simulations performed with LinB in the presence of 0.5 M solutions of NaCl, KCl, RbCl, and CsCl provided spatial distributions of the four alkali cations at the enzyme surface. The site located near the tunnel mouth, serving for substrates and products exchange, has been identified as the key region for specific ion effects on the activity of LinB. Two LinB mutants differing in cation binding at the tunnel mouth were constructed. Together with the wild type enzyme, they were functionally characterized by steady-state kinetics in aqueous solutions of alkali chloride salts. Kinetic analysis of the wild type and two mutants revealed that point mutations in the mouth lead to significantly modified interactions of the enzyme with its cognate substrate, and thus its catalytic properties.

In follow up study, effect of protonation state of the catalytic H272 of LinB on binding of halide anions and sodium cation inside the enzyme active site was explored (publication **P28**). Using stopped-flow fluorescence analysis and umbrella sampling free energy calculations, it was found out that the anion binding is ion-specific and follows the ordering $I^- > Br^- > Cl^-$. While deprotonation of H272 increased binding of anions in the access tunnel, the anionic ordering did not change with the change of the protonation state. Moreover, Na^+ easily entered the active site, provided the H272 residue is singly protonated, and replaces thus the missing proton. In contrast, Na^+ was strongly repelled from the active site containing the doubly protonated H272 residue.

7. Perspectives and research challenges

7.1. Analysis of hydration and flexibility of HLDs

Since hydration and flexibility have been identified as important determinants of HLD catalytic properties[82], detailed analysis of these properties by advanced experimental techniques is necessary in order to understand HLD catalysis more deeply at the molecular level. So far, hydration and flexibility of HLDs were predominantly investigated *in silico*[79,82,192,193]. The only experimental techniques employed were time-resolved and steady-state fluorescent spectroscopy. However, these techniques are based on the site-specific labeling of the protein by fluorescent probe or site-specific incorporation of fluorescent unnatural amino acids[82,109,110], and thus reflect only the hydration and flexibility of a particular protein region. Information about conformational changes of the whole protein, as well as information about dynamics of water in the hydration layers at the protein-surface is still largely missing. Next to the classical X-ray crystallography, NMR and molecular dynamics simulations, hydration and flexibility of HLDs can be studied by time-resolved X-ray crystallography[194–196], hydrogen-deuterium exchange coupled with mass spectrometry[197,198] and neutron crystallography[199,200]. These techniques may provide detailed information about conformational changes induced by diffusion of substrates into the HLDs nanocrystals in real-time, accessibility of HLDs to solvent and how solvent influences dynamics of HLD secondary structure, location of hydrogen atoms inside the enzyme active sites, and dynamics of water in hydration layers at the enzyme-surface interface. Although the preparation of HLD samples for time-resolved X-ray crystallography and neutron crystallography is time consuming and labor extensive[201], understanding of the relationships between HLDs catalysis and the enzyme hydration and flexibility pave the way towards the rational modification of their function.

7.2. Efficient identification and characterization of novel HLDs

Although more than 650 genes encoding putative HLDs have been identified in genome databases (Vanacek et al., unpublished data), only 24 of them have been, so far, successfully cloned, recombinantly expressed, and biochemically characterized. This imbalance between the exponential growth of DNA sequences in databases on one side and lack of the information about their function on the other side, highlights the necessity to develop an efficient platform allowing rational selection of attractive gene targets based on *in silico* screening of large pool of sequence entries, followed by experimental high-throughput characterization of the pre-selected protein hits. A platform for the rational selection of attractive gene targets should cover periodic searching of genetic databases, construction of multiple-sequence alignment, modelling of tertiary structures using homology modelling or *ab initio* predictions, identification of pockets and tunnels in these protein structures, docking or targeting of small molecules to the cavities, and clustering of proteins according to the their properties. The experimental high-throughput characterization of novel enzymes may employ either (i) the automated robotic pipetting system connected with the microtiter plate system where the putative enzymes will be cultivated and characterized for desired catalytic properties[202], or (ii) microfluidic devices coupled with florescence detection[203]. Microfluidic systems allows encapsulation of single cells into droplets which hold together cells, enzymes, substrates and products, enabling formation of much smaller micro-reactors compared to microtiter plates. Progress of enzymatic reaction is then monitored via formation of a fluorescent reaction product within a single droplet by microscope with a photomultiplier tube, or by using fluorescence-activated cell sorting system (FACS). The strategy combining database mining approach with the traditional low-throughput experimental testing has been recently employed for identification of 37 novel sequences of halohydrin dehalogenase

enzymes[204]. From this selection, 17 candidates were further biochemically characterized providing novel biocatalysts with unique substrate specificity, high activity and high thermostability[205], which demonstrates the feasibility of such an approach.

7.3. Development of high-throughput screening assay for HLDs

One challenge in directed evolution experiments is the coverage of a sufficiently large sequence space; that means the creation and analysis of as many variants as possible. Effective selection or screening methods should enable a fast, very accurate and targeted identification of desired biocatalysts out of libraries comprising usually 10^3-10^7 mutants. In the case of HLDs, all libraries constructed either by gene site saturation mutagenesis[159], saturation mutagenesis[12,79] or error-prone PCR[78] were screened by pH-indicator assay in microtiter plate format. The traditional microtiter plate screenings are medium-throughput (library size < 10^4), but are compatible with most analytical tools[206]. Other available screening techniques, such as cell surface display, *in vitro* compartmentalization, microfluidics or FACS, are high-throughput but require fluorogenic substrates[134,202,206]. The need to use a fluorescent molecule as a model substrate often leads to identification of hits that do not show the expected performance when exposed to the real/target substrate[202]. To expand the scope of ultra-high-throughput screenings, the current methods should move outside the limitations of fluorescence detection only. A promising development in this field is employing a microfluidics that has the potential to integrate alternative analytical tools. For example, the coupling of these screening technologies to mass spectrometry would dramatically widen the scope of enzyme reaction products that can be detected[206]. The development of such analytical tools will be very valuable to directed evolution of HLDs.

7.4. Identification of biological role of HLDs

Despite the fact that HLDs have been intensively studied for more than 20 years and have been isolated from many different environments and species, biological functions of most characterized enzymes remain elusive. The following approaches can be used to uncover physiological function of enzymes: (i) study of the total metabolite pool of a given organism or cell-type, known as metabolomics; (ii) activity-based metabolomic profiling; (iii) co-expression analysis for monitoring of changes in transcriptomic profiles to predict putative association between genes and defined metabolic function; (iv) gene knockout protocols allowing target gene disruption and subsequent assessment of the resulting phenotype or metabolic status to infer important information on enzyme function; (v) identification of co-purified small molecules associated with purified enzyme by X-ray crystallography or (vi) computational screening of putative substrates, products and intermediates based on structural determinants[207]. However, genetic methods are not always applicable; for example, in the cases when the candidate gene is essential for growth, the host organism is not genetically tractable or the cultivation of the organism is extremely slow or difficult to perform under laboratory conditions. The last-mentioned obstacle was previously observed when studying human pathogen *Mycobacterium tuberculosis* H37Rv in which genome three different HLD genes were identified[31,37]. Although genetic tools are relatively well developed for this pathogen, the slow growth rate (~ 20 h doubling time) of this organism makes gene knockout protocols cumbersome[207]. Identification of biological function of HLDs thus remains one of the greatest challenges for the future studies of this enzyme family.

8. References

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LIST OF ATTACHED PUBLICATIONS

P1 **Chaloupkova, R.**, Sykorova, J., Prokop, Z., Jesenska, A., Monincova, M., Pavlova, M., Nagata, Y., Damborsky, J., 2003: Modification of Activity and Specificity of Haloalkane Dehalogenase from *Sphingomonas paucimobilis* UT26 by Engineering of its Entrance Tunnel. *J. Biol. Chem.* 278: 52622-52628.

P2 Prokop, Z., Monincova, M., **Chaloupkova, R.**, Klvana, M., Janssen, D.B., Nagata, Y., Damborsky, J., 2003: Catalytic Mechanism of the Haloalkane Dehalogenase from *Sphingomonas paucimobilis* UT26. *J. Biol. Chem.* 278: 45094-45100.

P3 Sato, Y., Monincova, M., **Chaloupkova, R.**, Prokop, Z., Ohtsubo, Y., Minamisawa, K., Tsuda, M., Damborsky, J., Nagata, Y., 2005: Characterization of Haloalkane Dehalogenases of a New Structure and Substrate Specificity from Rhizobiaceae strains *Mesorhizobium loti* MAFF303099 and *Bradyrhizobium japonicum* USDA110. *Appl. Environ. Microbiol.* 71: 4372-4379.

P4 Jesenska, A., Pavlova, M., Strouhal, M., **Chaloupkova, R.**, Tesinska, I., Monincova, M., Prokop, Z., Bartos, M., Pavlik, I., Rychlik, I., Möbius, P., Nagata, Y., Damborsky, J., 2005: Cloning, Biochemical Properties, and Distribution of Mycobacterial Haloalkane Dehalogenases. *Appl. Environ. Microbiol.* 71: 6736-6745.

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P8 Pavlova, M., Klvana M., Prokop, Z., **Chaloupkova, R.**, Banas, P., Otyepka, M., Wade, R., Tsuda, M., Nagata, Y., Damborsky J., 2009: Redesigning Dehalogenase Access Tunnels as a Strategy for Degrading an Antropogenic Substrate. *Nat. Chem. Biol.* 5: 727-733.

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P10 Prokop, Z., Sato, Y., Brezovsky, J., Mozga, T., **Chaloupkova, R.**, Koudelakova, T., Jerabek, P., Stepankova, V., Natsume, R., van Leeuwen, J.G.E., Janssen, D.B., Florian, J., Nagata, Y., Senda, T., Damborský J., 2010: Enantioselectivity of Haloalkane Dehalogenases and its Modulation by Surface Loop Engineering. *Angew. Chem. Int. Ed. Engl.* 49:6111-6115.

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P23 Stepankova, V., Paterova, J., Damborsky, J., Jungwirth, P., **Chaloupkova, R.**, Heyda, J., 2013: Cation-Specific Effects on Enzymatic Catalysis Driven by Interactions at the Tunnel Mouth. *J. Phys. Chem. B* 117: 6394-6402.

P24 Tratsiak, K., Degtjarik, O., Drienovska, I., Chrast, L., Rezacova, P., Kuty, M., **Chaloupkova, R.**, Damborsky, J., Kuta Smatanova, I., 2013: Crystallographic Analysis of New Psychrophilic Haloalkane Dehalogenases: DpcA from Psychrobacter cryohalolentis K5 and DmxA from Marinobacter sp. ELB17. *Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun.* 69: 683-688.

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P29 Lahoda, M., Mesters, J. R., Stsiapanava, A., **Chaloupkova, R.**, Kuty, M., Damborsky, J., Kuta Smatanova, I., 2014: Crystallographic Analysis of 1,2,3–Trichloropropane Biodegradation by Haloalkane Dehalogenase DhaA31. *Acta Crystallogr. D Biol. Crystallogr.* 70: 209-217.

P30 Stepankova, V., Vanacek, P., Damborsky, J., **Chaloupkova, R.**, 2014: Comparison of Catalysis by Haloalkane Dehalogenases in Aqueous Solutions of Deep Eutectic and Organic Solvents. *Green Chem.* 16: 2754-2761.

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P32 **Chaloupkova, R.**, Prudnikova, T., Rezacova, P., Prokop, Z., Koudelakova, T., Daniel, L., Brezovsky, J., Ikeda-Ohtsubo, W., Sato, Y., Kuty, M., Nagata, Y., Kuta Smatanova, I., Damborsky, J., 2014: Structural and Functional Analysis of a Novel Haloalkane Dehalogenase with Two Halide-Binding Sites. *Acta Crystallogr. D Biol. Crystallogr.* 70: 1884-1897.

P33 Liskova, V., Bednar, D., Prudnikova, T., Rezacova, P., Koudelakova, T., Sebestova, E., Kuta Smatanova, I., Brezovsky, J., **Chaloupkova, R.**, Damborsky, J., 2015: Balancing the stability-activity trade-off by fine-tuning dehalogenase access tunnels. *ChemCatChem* 7: 648-659.

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P35 Bednar, D., Beerens, K., Sebestova, E., Bendl, J., Khare, S., **Chaloupkova, R.**, Prokop, Z., Brezovsky, J., Baker, D., Damborsky, J., 2015: FireProt: Energy- and Evolution-Based Computational Design of Thermostable Multiple-Point Mutants. *PLOS Comput. Biol.* 11: e1004556.

AUTHOR CONTRIBUTION

I declare that my contribution to the above publications was as:

P1 Enzyme expression and purification, CD spectroscopy measurements, activity measurements, steady-state enzyme kinetics, PCA analysis of substrate specifity data, data interpretation, writing of the manuscript.

P2 Enzyme expression and purification, steady-state enzyme kinetics, inhibition kinetics, writing of the manuscript.

P3 CD spectroscopy measurements, analysis of secondary structure content of tested enzymes, writing of the manuscript.

P4 CD spectroscopy measurements, thermostability measurements, data analysis, writing of the manuscript.

P5 CD spectroscopy measurements, thermostability measurements, data analysis, writing of the manuscript.

P6 Enzyme expression and purification, writing of the manuscript.

P7 CD spectroscopy measurements, thermostability measurements, data analysis, writing of the manuscript.

P8 CD spectroscopy measurements, solvent kinetic isotopic effect measurements, data analysis, writing of the manuscript.

P9 CD spectroscopy measurements, thermostability measurements, data analysis, writing of the manuscript.

P10 Thermodynamic analysis of enantioselectivity of tested enzyme variants, data interpretation, writing of the manuscript.

P11 CD spectroscopy measurements, thermostability measurements, data analysis, writing of the manuscript.

P12 Enzyme expression and purification, writing of the manuscript.

P13 Enzyme expression and purification, writing of the manuscript.

P14 Enzyme expression and purification, writing of the manuscript.

P15 Enzyme expression and purification, CD spectroscopy measurements, analysis of secondary structure content of tested enzymes, thermostability measurements, activity measurements, enantioselectivity measurements, thermodynamic analysis of enzyme enantioselectivity, size-exclusion chromatography measurements, data interpretation, writing of the manuscript.

P16 Experimental design, data interpretation, writing of the manuscript.

P17 Thermostability measurements, writing of the manuscript.

P18 CD spectroscopy measurements, DSC measurements, data analysis, writing of the manuscript.

P19 Enzyme expression and purification, writing of the manuscript.

P20 Experimental design, data interpretation, writing of the manuscript.

P21 CD spectroscopy measurements, thermostability measurements, data analysis, writing of the manuscript.

P22 Experimental design, data interpretation, writing of the manuscript.

P23 Experimental design, data interpretation, writing of the manuscript.

P24 Enzyme expression and purification, writing of the manuscript.

P25 Enzyme expression and purification, labelling, writing of the manuscript.

P26 Experimental design, CD spectroscopy measurements, thermostability measurements, data interpretation, writing of the manuscript.

P27 Writing of the manuscript.

P28 Experimental design, data interpretation, writing of the manuscript.

P29 Enzyme expression and purification, writing of the manuscript.

P30 Experimental design, data interpretation, writing of the manuscript.

P31 CD spectroscopy measurements, thermostability measurements, data analysis, writing of the manuscript.

P32 Enzyme expression and purification, CD spectroscopy measurements, thermostability measurements, size-exclusion chromatography measurements, activity measurements, steady-state enzyme kinetics, data interpretation, writing of the manuscript.

P33 Experimental design, data interpretation, writing of the manuscript.

P34 CD spectroscopy measurements, thermodynamic analysis of enzyme enantioselectivity, data interpretation, writing of the manuscript.

P35 CD spectroscopy measurements, thermostability measurements, data analysis, writing of the manuscript.

CURRICULUM VITAE

Contact information

Name: Radka Chaloupkova
Address: Kamenice 5/A13, 625 00 Brno, Czech Republic
Email: radka @chemi.muni.cz

Personal information

Date of Birth: July 8, 1978
Place of Birth: Rychnov nad Kněžnou, Czech Republic
Nationality: Czech

Education and qualification

2008	Ph.D., Department of Biochemistry, Biomolecular Chemistry, Faculty of Science, Masaryk University, Brno
2001	MSc., **Department of Theoretical and Physical Chemistry**, Faculty of Science, Masaryk University, Brno

Employment

2006-now	Loschmidt Laboratories, Department of Experimental Biology and Research Centre for Toxic Compounds in the Environment, Faculty of Science, Masaryk University, Brno

Research activities

circular dichroism spectroscopy, fluorescence spectroscopy, X-ray crystallography, isothermal titration calorimetry, differential scanning calorimetry, steady-state enzyme kinetics, thermodynamic analysis of enzyme catalytic activity and enantioselectivity, size-exclusion chromatography, light scattering, intrinsic viscosity, medium engineering, protein engineering

Awards

2012	Award for the Best Poster Presentation, 9th International Conference on Protein Stabilization
2008	II.-IV. prize, poster, EMBO-ESF Conference
2007	Award of the Rector of the Masaryk University
2006	I. prize, lecture at students' competition, Sigma-Aldrich Meeting of Young Molecular Biologists, Biochemists and Chemists
2005	Award of the Dean of the Faculty of Science, Masaryk University

Research projects

2016-2018	Grant Agency of the Czech Republic Structural Basis for the Emergence of New Enzymatic Activities; 16-24223S; principal investigator
2012-2016	Grant Agency of the Czech Republic Structure-Functional Relationships of Haloalkane Dehalogenases; P207/12/0775; principal investigator

2013-2015 Grant of Ministry of Education, Youth and Sports of the Czech Republic
Employment of Young Scientists to Promote International Collaboration;
CZ.1.07/2.3.00/30.0037; project team member

2008-2012 Grant Agency of the Czech Republic
Specific Ion Effects for Proteins in Solutions and Related Biologically Relevant Systems; 203/08/0114; project team member

2006-2011 Grant of Ministry of Education, Youth and Sports of the Czech Republic
Center for Biocatalysis and Biotransformation; LC06010; project team member

Research fellowships

2015 Research Stay at the Diamond Light Source Ltd., Diamond House, Harwell Science and Innovation Campus (January and October 2015), (Didcot, Oxfordshire, UK)

2014 Research Stay at the Diamond Light Source Ltd., Diamond House, Harwell Science and Innovation Campus (January 2014), (Didcot, Oxfordshire, UK)

2013 Research Stay at the Diamond Light Source Ltd., Diamond House, Harwell Science and Innovation Campus (November-December 2013), (Didcot, Oxfordshire, GB)

2003 Marie Curie Fellowship, EU (September-December 2003), Research Stay at the Biophysical and Bioanalytical Chemistry Group, University of Warwick, (Coventry, GB)

International and national courses

2013 EMBO Laboratory Management Course for Postdocs (3-5 December 2013),
(Leimen, D)

2010 Binding and Kinetics for Experimental Biologists (22-26 November 2010)
Department of Experimental Biology, Faculty of Science, Masaryk University (Brno, CZ)

2008 Biomolecules: Physical Principles and Mechanisms (1-5 November 2008)
Institute of Organic Chemistry and Biochemistry, Academy of Sciences, (Prague, CZ)

2007 Applied Biocatalysis: Stereoselective and Environmentally-friendly Reactions Catalyzed by Enzymes (28 April - 3 May 2007)
COST D25 Training School (Certosa di Pontignano-Siena, IT)

2004 EMBO Young Investigator Programme PhD Course (15-21 September 2004)
EMBL (Heidelberg, D)

2004 Spectroscopic Techniques and Their Applications
(29 August - 3 September 2004)
J. Heyrovsky Institute of Physical Chemistry, Academy of Sciences
(Prague, CZ)

2003 EPSRC Circular Dichroism Summer School (8-12 September 2003)
University of Warwick (Coventry, GB)

Supervision of students

2016-now Gabriela Dankova, Doctoral Thesis, Structural analysis of selected growth factors, Department of Experimental Biology, Faculty of Science, Masaryk University, Brno

2014-now Petra Babkova, Doctoral Thesis, Structure-Function Relationships and Evolution of Haloalkane Dehalogenases Studied by Ancestral Sequence Reconstruction, Environmental Chemistry, Faculty of Science, Masaryk University, Brno

2013-now Klaudia Sarmirova, Doctoral Thesis, Molecular Basis of Haloakane Dehalogenase Activity and Stability at Extreme Conditions, Department of Experimental Biology, Faculty of Science, Masaryk University, Brno

2012-now Lukas Chrast, Doctoral Thesis, Metabolic Engineering of Microorganisms for Degradation of Toxic Halogenated Compounds and Production of Optically Pure Compounds, Department of Experimental Biology, Faculty of Science, Masaryk University, Brno

2011-now Veronika Liskova, Doctoral Thesis, Study of Oligomerization of Proteins as an Important Factor for Development of Alzheimer and Parkinson Disease, Department of Experimental Biology, Faculty of Science, Masaryk University, Brno

2008-2013 Veronika Stepankova, Doctoral Thesis, Haloalkane Dehalogenases in Non-conventional Media, Department of Biochemistry, Faculty of Science, Masaryk University, Brno (Supervisor-specialist)

2012-2014 Petra Szelcsanyiova, Diploma Thesis, Biochemical Characterization of Haloalkane Dehalogenases with Modified Tunnels, Department of Microbiology, Faculty of Science, Masaryk University, Brno

2010-2012 Ivana Drienovska, Diploma Thesis, Structural-Functional Basis of Psychrotolerance of Haloalkane Dehalogenase Enzyme Isolated from Extremophilic Organism, Department of Biochemistry, Faculty of Science, Masaryk University, Brno

2010-2012 Lukas Chrast, Diploma Thesis, Characterization of Novel Biocatalysts from Psychrophilic and Thermophilic Bacteria, Department of Experimental Biology, Faculty of Science, Masaryk University, Brno

2015-2016 Klara Markova, Bachelor Thesis, Crystallization and Structural Characterization of Stable Variants of Haloalkane Dehalogenase DhaA, Department of Experimental Biology, Faculty of Science, Masaryk University, Brno

2011-2012 Petra Szelcsanyiova, Bachelor Thesis, Screening of Isolated or Newly Developed Biocatalysts with High Stability and Activity *in vitro* and *in vivo*, Department of Microbiology, Faculty of Science, Masaryk University, Brno

2009-2010 Simona Hankeova, Bachelor Thesis, Effect of Ions on Catalytic Function of Hydrolytic Enzymes, Department of Experimental Biology, Faculty of Science, Masaryk University, Brno

2009-2010 Ivana Drienovska, Bachelor Thesis, New Biocatalysts from Extremophilic Bacteria, Department of Biochemistry, Faculty of Science, Masaryk University, Brno

2015-2016 Daniel Pluskal, SOČ (School Scientific Activity), Analysis of Catalytic Amino Acids of Haloalkane Dehalogenase, Luciferase and Their Reconstructed Ancestor, Grammar School Brno-Reckovice, Brno

Teaching activities

2013-now Protein Engineering Course (Bi7410), Faculty of Science, Masaryk University

2011-now Seminar of Loschmidt Laboratories (Bi7893), Faculty of Science, Masaryk University

2011-now Seminar of Research Teams of Loschmidt Laboratories (Bi7894), Faculty of Science, Masaryk University

2010-now Summer School of Protein Engineering (Bi9280), Faculty of Science, Masaryk University

2009-2011 Protein Preparation and Characterization II – practice (Bi8858c), Faculty of Science, Masaryk University

Publication activities
52 scientific articles (3 articles as first author, 8 articles as corresponding author), 4 book chapters, 1 international patent, 1 patent application

WEB OF SCIENCE CITATION REPORT (September 12, 2016)
Results found: 52
Sum of the Times Cited: 816
Sum of Times Cited without self-citations: 673
Citing Articles: 512
Citing Articles without self-citations: 474
Average Citations per Item: 15.69
h-index: 16

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BOOK CHAPTERS

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