FEBS 2023 ADVANCED COURSE

Computational Approaches to Understanding and Engineering Enzyme Catalysis

Book of abstracts

Department of Chemistry
University of Zagreb Faculty of Science

September 25th–29th, 2023
Zagreb, Croatia, EU

FEBS 2023
ADVANCED COURSE

Computational Approaches to Understanding and Engineering Enzyme Catalysis

Book of abstracts

Department of Chemistry, Faculty of Science
University of Zagreb

September 25th–29th, 2023
Zagreb, Croatia, EU

Foreword

The 2nd edition of the FEBS Advanced Practical and Lecture Course on Computational Approaches to Understanding and Engineering Enzyme Catalysis, hosted in Zagreb, Croatia, serves as a pivotal platform for the dissemination of crucial in silico tools applicable to experimental research projects. This event primarily targets Ph.D. students and postdocs engaged in ongoing research endeavors that necessitate a deep understanding and utilization of the tools presented during the course.

Our program revolves around four central tutorial and lecture topics:

(i) European Bioinformatics Institute (EMBL-EBI) Enzyme Resources: This segment encompasses valuable enzyme resources provided by EMBL-EBI. Prof. Dame Janet Thornton and Dr. Deepti Gupta will provide comprehensive coverage of this topic.

(ii) Molecular dynamics simulation as a tool in protein engineering: Prof. Chris Oostenbrink will delve into the application of molecular dynamics simulation in the realm of protein engineering.

(iii) Computational tools for enzyme design and engineering: Prof. Jiří Damborský and Dr. David Bednář, Ph.D., will guide participants through the world of computational tools tailored for designing and engineering enzymes.

(iv) De Novo Protein Design: Prof. David Baker and Research Asst. Prof. Ajasja Ljubetič will shed light on the exciting field of de novo protein design.

Beyond these four main combined lecture and tutorial topics, our program further enriches participants' knowledge through lectures by esteemed experts. Professor Ita Gruić-Sovulj will explore the kinetic analysis of enzyme-catalyzed reactions, while Prof. Kenneth A. Johnson will introduce the versatile KinTek Explorer software, an invaluable resource for simulation and data fitting in the realm of enzyme kinetics.

We are also honored to host Prof. Don Hilvert, who will share insights into the design of proteins with customized catalytic properties. Dr. Marc Van der Kamp and Assoc. Prof. Jernej Stare will provide in-depth insights into the QM/MM and EVB approaches for simulating enzyme-catalyzed reactions. Furthermore, Asst Prof. Tina Perica will unravel the intricate world of allosteric pathways in protein function, while Assoc. Prof. Per-Olof Syrén will enlighten us on novel enzyme engineering strategies, particularly their applications in biopolymer science.

Our comprehensive program aims to bridge the gap between computational and experimental research, facilitating collaboration and knowledge exchange among participants. We look forward to an engaging and enlightening experience during this course.

The Organizing Committee

Program of the *FEBS 2023 Advanced Course*

Day	Time	Lectures	Tutorials/Demonstrations	Poster Session
	Chairs: Aleksandra Maršavelski and Tomica Hrenar			
25th September 2023 **Monday**	9:00-9:50 **REGISTRATION**			
	9:50-10:10 **Opening words (A1 lecture hall)**			
	10:10–11:00	**Professor Dame Janet Thornton** Computational Enzymology - Enzyme Reactions and active sites in 1D, 2D and 3D		
	11:00–11:10	**Q&A**		
	11:10-12:00	**Professor Ita Gruić-Sovulj** Pre-steady and steady state kinetics of enzyme catalysed reactions		
	12:00–12:10	**Q&A**		
	12:10–13:30 **Lunch break**			
	13:30–14:20	**Professor Kenneth A. Johnson** KinTek Explorer software: explore key kinetic concepts		
	14:20–14:30	**Q&A**		
	14:30–16:30		**Dr. Deepti Gupta** Tutorial on using PDBe and PDBe-KB tools	
	16:30-17:00 **Coffee break**			
	17:00–19:00			**Two-minute talks and Poster presentation**

Day	Time	Lectures	Tutorials/Demonstrations	Poster Session
26th September 2023 **Tuesday**	**Chairs: Outi Lampela, Tiila-Riikka Kiema and Rikkert Wierenga**			
	9:30–10:20	**Assistant Professor Tina Perica** Allosteric pathways in protein function		
	10:20–10:30	**Q&A**		
	10:30–11:20	**Professor Chris Oostenbrink** Molecular dynamics simulation as a tool in protein engineering		
	11:20–11:30	**Q&A**		
	11:30–13:00 **Lunch break**			
	13:00–15:00		**Professor Chris Oostenbrink** Tutorial on MD simulations	
	15:00–16:30		**Professor Chris Oostenbrink** Tutorial on MD simulations; Analysis of Protein Conformational Changes	
	16:30–17:00 **Coffee break and discussion**			
	17:00–18:30		**Professor Chris Oostenbrink** Analysis of Protein Conformational Changes	

Day	Time	Lectures	Tutorials/Demonstrations	Poster Session
27th September 2023 **Wednesday**	**Chairs: Outi Lampela, Tiila-Riikka Kiema and Rikkert Wierenga**			
	9:00–10:30	**Research Assistant Profesor Ajasja Ljubetič** Introduction to de novo protein design using PyRosetta and deep learning methods.		
	10:30–10:50	**Professor Antonio Díaz Quintana,** FEBS-MIC, FEBS opportunities for scientists in molecular life sciences		
	10:50–12:30		**Research Assistant Profesor Ajasja Ljubetič** Parametric design of helical bundles with PyRosetta	
	12:30–14:00 **Lunch break**			
	14:00–15:30		**Research Assistant Professor Ajasja Ljubetič** Design of side-chains using ProteinMPNN and AlphaFold2	
	15:30–17:00		**Research Assistant Professor Ajasja Ljubetič** Designing proteins using Rosetta Fold Diffusion	
	17:00–18:00 **Coffee break**			
	18:00–18:50	**On-line lecture Professor David Baker** Design of new protein functions using deep learning		

	18:50–19:00	**Q&A**		
	19:00 Departure			
	20:00 Social event, Dinner in town, Johann Franck, *Trg bana Josipa Jelačića 9, Zagreb*			

Day	Time	Lectures	Tutorials/Demonstrations	Poster Session
28th September 2023 **Thursday**	**Chairs: Outi Lampela, Tiila-Riikka Kiema and Rikkert Wierenga**			
	9:30–10:20	**Dr. Marc Van der Kamp** The use of (QM/MM) biomolecular simulation to understand enzyme-catalyzed reactions: activity and selectivity		
	10:20–10:30	**Q&A**		
	10:30–11:20	**Profesor Jernej Stare** Reaction Pathway Sampling by Empirical Valence Bond Simulation		
	11:20–11:30	**Q&A**		
	11:30–13:00 **Lunch break**			
	13:00–13:50	**Professor Jiri Damborsky** Computational tools for designing and engineering enzymes		
	13:50–14:00	**Q&A**		
	14:00–15:30		**Professor Jiri Damborsky/ Mgr. David Bednář, Ph.D.**	

			Engineering Protein Stability (FireProt; FireProt^{ASR}and FireProt^{DB})	
Day	**Time**	**Lectures**	**Tutorials/Demonstrations**	**Poster Session**
28th September 2023 **Thursday**	15:30–17:30		**Professor Jiri Damborsky/ Mgr. David Bednář, Ph.D.** Identification of Enzyme Tunnels and Ligand Pathways - Caver Web and CaverDock; Identification of Hot Spots and Design of Smart Libraries - HotSpot Wizard	
	17:30–18:00 **Coffee break**			
	18:00–19:00	**Q&A session** on all tutorials and lectures during the course		
29th September 2023 **Friday**	**Chair: Aleksandra Maršavelski**			
	9:30–10:20	**Professor Per-Olof Syrén** Novel enzyme engineering strategies for applications in biopolymer science		
	10:20–10:30	**Q&A**		
	10:30–11:20	**Professor Donald Hilvert** Designing proteins with customized catalytic properties		
	11:20–11:30	**Q&A**		
	11:30–12:00	**The Best Presentation and FEBS Open Bio Poster Prize Award** **Concluding remarks Aleksandra Maršavelski and Rikkert Wierenga**		
Lunch and Departure				

Table of contents

List of lectures

1. **Professor Dame Janet Thornton**
   Computational Enzymology - Enzyme Reactions and active sites in 1D, 2D and 3D
2. **Professor Ita Gruić-Sovulj**
   Pre-steady and steady state kinetics of enzyme catalysed reactions
3. **Professor Kenneth A. Johnson**
   KinTek Explorer software: explore key kinetic concepts
4. **Assistant Professor Tina Perica**
   Allosteric pathways in protein function
5. **Professor Chris Oostenbrink**
   Molecular dynamics simulation as a tool in protein engineering
6. **Research Assistant Profesor Ajasja Ljubetič**
   Introduction to de novo protein design using PyRosetta and deep learning methods
7. **Professor Antonio Díaz Quintana**
   FEBS-MIC, FEBS opportunities for scientists in molecular life sciences
8. **Professor David Baker**
   Design of new protein functions using deep learning
9. **Dr. Marc Van der Kamp**
   The use of (QM/MM) biomolecular simulation to understand enzyme-catalyzed reactions: activity and selectivity
10. **Profesor Jernej Stare**
    Reaction Pathway Sampling by Empirical Valence Bond Simulation
11. **Professor Jiri Damborsky**
    Computational tools for designing and engineering enzymes
12. **Professor Per-Olof Syrén**
    Novel enzyme engineering strategies for applications in biopolymer science
13. **Professor Donald Hilvert**
    Designing proteins with customized catalytic properties

List of computer class tutorials

1. **Dr. Deepti Gupta**
   Tutorial on using PDBe and PDBe-KB tools

2. **Professor Chris Oostenbrink**
   Tutorial on MD simulations
   Analysis of Protein Conformational Changes

3. **Research Assistant Profesor Ajasja Ljubetič**
   Parametric design of helical bundles with PyRosetta
   Design of side-chains using ProteinMPNN and AlphaFold2
   Designing proteins using Rosetta Fold Diffusion

4. **Professor Jiri Damborsky & Mgr. David Bednář, Ph.D.**
   Engineering Protein Stability (FireProt; FireProtASRand FireProtDB)
   Identification of Enzyme Tunnels and Ligand Pathways - Caver Web and CaverDock
   Identification of Hot Spots and Design of Smart Libraries - HotSpot Wizard

Posters

P 1. Unlocking the specificity determinants of NS-SDR epimerases through rational design

Carlos Josué Alvarez Quispe, Koen Beerens, Tom Desmet

carlosjosue.alvarezquispe@ugent.be

Ghent University, Ghent, Belgium

Nucleotide sugar epimerases (NS-SDR epimerases)[1] form a very interesting class of enzymes, as they can invert the configuration of a specific hydroxyl group through a single reaction without prior activation or protection steps. As a result, an unusual sugar structure can be generated from a more common counterpart, leading to new properties and potential applications in the food and pharma industries. In many cases, however, the desired combination of regioselectivity (e.g. C3-position) and substrate selectivity (e.g. UDP-Glc) is not present in one and the same enzyme (e.g. to generate UDP-Allose), which limits the number of routes that are available for large-scale production processes. Recently, the discovery of a novel and highly promiscuous Gal4E (UDP-galactose 4-epimerase) from *Pyrococcus horikoshii* [2] opens an opportunity for epimerase engineering. This novel group of enzymes displayed an unprecedented specificity on guanosine diphosphate (GDP) sugars. In addition, we confirmed that it accepts a variety of other NDP-sugars including L-sugars moieties. This unknown specificity suggests a close evolutionary relationship between this group of promiscuous Gal4E enzymes and the GM35E (GDP-mannose 3,5-epimerase). In light of this, our approach involves employing molecular dynamics simulations and ancestral sequence reconstruction techniques to enhance our comprehension of the underlying structure-function relationships within these enzymes. Based on that information, we will design either a new-to-nature UDP-Glc 3,5-epimerase or GDP-Man 4-epimerase by combining features of existent Gal4E and GM35E. Ultimately, this endeavour aims to facilitate the creation of customized biocatalysts capable of synthesizing a diverse spectrum of rare sugars and their derivatives.

References:

[1] Da Costa M, Gevaert O, Van Overtveldt S, Lange J, Joosten HJ, Desmet T, Beerens K: Structure-function relationships in NDP-sugar active SDR enzymes: Fingerprints for functional annotation and enzyme engineering. *Biotechnol Adv* 2021, 48:107705.

[2] Alvarez Quispe C, Da Costa M, Beerens K, Desmet T: Exploration of archaeal nucleotide sugar epimerases unveils a new and highly promiscuous GDP-Gal4E subgroup. Curr Res Biotechnol 2022, 4:350–358.

P 2. Enhancing microbial polylactic acid degradation for recycling purposes

Margaux Aubel[1], Erich Bornberg-Bauer[1], Aleksandra Marsavelski[2]

m_aube01@uni-muenster.de

[1] Institute for Evolution and Biodiversity, University of Münster, Münster, Germany
[2] Department of Chemistry, Faculty of Science, University of Zagreb, Zagreb, Croatia

Starting with the industrialisation, chemical factories have released many xenobiotics to the environment over the last decades posing a new kind of evolutionary pressure on different organisms. Microorganisms in particular have demonstrated the ability to quickly evolve new functions in response to otherwise undegradable or toxic substances like plastics. For example, polylactic acid (PLA) is a biodegradable polyester with high potential for applications in many fields. Despite its promises, the biodegradability of PLA is low in natural environments and limits its use as a sustainable alternative to conventional plastics. However, a metagenomic screen identified an esterase with hydrolytic activity towards PLA. [1] Using molecular dynamics simulations, we aim to identify residues that can be mutated in order to improve the enzymatic specificity and enzyme thermostability. As an additional approach, we have reconstructed ancestral sequences of the PLA degrading enzymes. The reconstructed ancestral sequences will be optimised with ZymCTRL [2], a machine learning approach using protein language models. Three sequences of each approach, a) rational mutation, b) ancestral sequence reconstruction, and c) ZymCTRL, will be screened experimentally for activity and stability in the end.

References:

[1] M. Hajighasemi, B. P. Nocek, A. Tchigvintsev, G. Brown, R. Flick, X. Xu, H. Cui, T. Hai, A. Joachimiak, P. N. Golyshin, A. Savchenko, E. A. Edwards, and A. F. Yakunin. Biochemical and Structural Insights into Enzymatic Depolymerization of Polylactic Acid and Other Polyesters by Microbial Carboxylesterases. Biomacromolecules 2016, 17 (6), 2027-2039.

[2] G. Munsamy, S. Lindner, P. Lorenz, N. Ferruz. ZymCTRL: a conditional language model for the controllable generation of artificial enzymes. Machine Learning for Structural Biology Workshop, NeurIPS 2022.

P 3. Esterase sequence composition patterns as inspiration for the design of short catalytic peptides

Marko Babić, Daniela Kalafatović

marko.babic@uniri.hr

University of Rijeka, Department of Biotechnology, Croatia

Efficient ester hydrolysis catalysts have been a subject of significant interest in biomedical research and biomaterials engineering. Although peptide-based catalysts have been explored, their catalytic efficiency is limited by conformational heterogeneity compared to natural enzymes.

Here, we present an analysis of the primary sequence composition of 974 enzymes performing ester hydrolysis (EC 3.1.), which has uncovered highly conserved catalytic sites with distinct positional patterns. This study focuses on three critical features of enzyme efficiency: the catalytic residues identified by mechanism studies, the spatial geometry of these residues, and the conserved chemical properties of catalytic microenvironments. Leveraging this knowledge, we aimed to accelerate the discovery of short catalytic peptides for ester hydrolysis.

Our recent publication detailed the catalytic microenvironments present in the primary sequence of these enzymes[1]. Building on this, we analyzed the 3-D microenvironment of these enzymes using PyMol to measure interatomic distances between key residues from PDB structures and AlphaFold predictions using the previously constructed dataset of 974 enzymes. We compared the microenvironments of all 974 proteins using a modified alignment method that centers residues based on catalytic triad members instead of genetic properties or α-carbons, as done with other structural or sequence alignment methods. The analysis focused on distances and angles between the nucleophile, base and acid residues and the hydrogens with which they are connected through hydrogen bonds. We performed a visual inspection and marked and measured the clusters of atoms N, S, and O, combined with cyclic residue structures towards the active residues.

The results of our analysis revealed highly conserved catalytic residues and a pattern in residue chemical property placement in the space near the catalytic residues. These findings suggest that certain residues besides the catalytic triad play important roles in peptide design and offer new candidates for improving catalytic efficiency, addressing the conformational heterogeneity problem in peptide-based catalysts

References:

[1] Babić M, Janković P, Marchesan S, Mauša G, Kalafatovic D. Esterase Sequence Composition Patterns for the Identification of Catalytic Triad Microenvironment Motifs. J Chem Inf Model. 2022 Dec 26;62(24):6398–410.

P 4. Discovery and structural characterization of a thermostable bacterial monoamine oxidase

Lorenzo Basile[1], Lars L. Santema[2], Chiara Poli[1], Marco W. Fraaije[2], Claudia Binda[1]

lorenzo.basile01@universitadipavia.it

[1] Department of Biology and Biotechnology, Università degli Studi di Pavia, Pavia, 27100, Italy
[2] Molecular Enzymology, University of Groningen, Nijenborgh 4, 9747AG Groningen, The Netherlands

Biogenic amines are organic bases with one or more nitrogen groups[1]. Their biological roles are numerous, and they play important roles in various processes such as antioxidants and neurotransmitters[2&3]. Besides their biological relevance, their detection is of economic importance as they are widely used as marker for the quality of food[4]. Biosensors based on enzymes offer a rapid and economical methods for the detection of amines from microorganisms[5]. Amine oxidases are excellent tools for sensing of amines and can be used for the detection of amines. Apart from their use in biosensors, amine oxidases are also attractive biocatalysts. It has been demonstrated that amine oxidases can be used for the generation of various high value pharma compounds, allowing the synthesis of enantiopure amines[6].

Clearly, MAOs have considerable biotechnological potential, and homologs are highly sought after for their potential use. Bacterial genomes harbor genes encoding (putative) MAOs. While the precise biological function of MAOs in prokaryotes is unknown, probably they play a role in degradation pathways, utilizing amines as nitrogen source[7]. Thus, bacteria form a potential source for new homologues. Such bacterial homologs have an attractive feature when compared with eukaryotic MAOs: the expression in bacterial hosts provides easy access to these potential biocatalysts and enables the use of effective enzyme engineering approaches. Thus, thermophilic bacteria form a promising source for thermophilic MAO homologs[8]. Herein, using the sequence of putrescine oxidase from Rhodococcus erythropolis (PuORh)[9] as query, a thermostable MAO was discovered from a thermophilic Thermoanaero¬bacterales bacterium (MAOTb).

MAOTb is highly thermostable with melting temperatures above 80 ^{o}C and is well expressed in Escherichia coli. Substrate screening revealed that the oxidase is most efficient with n-alkylamines with n-heptylamine being the best substrate. Pre-steady state kinetic analysis shows that reduced MAOTb rapidly reacts with molecular oxygen confirming that it is a bona fide oxidase. The crystal structure of MAOTb was resolved at 1.5 Å and showed an exceptionally high similarity with the two human monoamine oxidases, MAO A and MAO B. The active site of MAOTb resembles most the architecture of human MAO A, including the cysteinyl protein-FAD linkage. Yet, the bacterial MAO lacks the C-terminus domain found in human MAOs which explains why it is expressed and purified as soluble protein, while the mammalian counterparts are anchored to the membrane through a their □-helix. MAOTb also displays a slightly different active site access tunnel which may explain the specificity towards long aliphatic amines. The crystal structures of MAOTb bound to putrescine and benzylamine were resolved at 2.2 Å. These structures showed very interesting features that could help us to engineer some of MAOTb active site residues. With being an easy to express, thermostable enzyme for which a high-resolution structure was elucidated, this bacterial MAO may develop into a valuable biocatalyst for synthetic chemistry or biosensing

References:

[1] Santos, M. H. A. Biogenic amines: their importance in foods. International Journal of Food Microbiology 1996, 29(2–3), 213–231.

[2] Madeo, F., Eisenberg, T., Pietrocola, F., & Kroemer, G. Spermidine in health and disease. Science 2018, 359(6374).

[3] Evans, P. Biogenic Amines in the Insect Nervous System. Advances in Insect Physiology 1980, 317–473.

[4] Brink, B. T., Damink, C., Joosten, H., & Veld, J. H. I. ’. Occurrence and formation of biologically active amines in foods. International Journal of Food Microbiology 1990, 11(1), 73–84.

[5] Önal, A. Food Chemistry 2007, 103, pp. 1475-1486.

[6] Ghislieri, D., Houghton, D., Green, A. R., Willies, S. C., & Turner, N. J. Monoamine Oxidase (MAO-N) Catalyzed Deracemization of Tetrahydro-β-carbolines: Substrate Dependent Switch in Enantioselectivity. ACS Catalysis 2013, 3(12), 2869–2872.

[7] Muellers, S. N., Tararina, M. A., Kuzmanovic, U., Galagan, J. E., & Allen, K. N. Structural Insights into the Substrate Range of a Bacterial Monoamine Oxidase. Biochemistry 2023, 62(3), 851–862.

[8] Lasa, I. R., & Berenguer, J. Thermophilic enzymes and their biotechnological potential. Microbiologia 1993, 9(2), 77–89.

[9] Van Hellemond, E. W., Van Dijk, M., Heuts, D. P. H. M., Janssen, D. B., & Fraaije, M. W. Discovery and characterization of a putrescine oxidase from Rhodococcus erythropolis NCIMB 11540. Applied Microbiology and Biotechnology 2008, 78(3), 455–463.

P 5. Enzyme engineering of fructosyl peptide oxidase to widen its active site access tunnel and improve its thermal stability

Shapla Bhattacharya, Rossella Catagna, Hajar (Nika) Estiri, Jhon Alex Buitrago, Alfonso Gautieri, Emilio Parisini

Latvian Institute of Organic Synthesis, Aizkraukles 21, Riga, Latvia

Enzyme engineering is a tailoring process that allows the modification of naturally-occurring enzymes to provide them with improved catalytic efficiency, stability or specificity. By introducing partial modifications to their sequence and to their structural features, enzyme engineering can transform natural enzymes into more efficient, specific, resistant biocatalysts and render them suitable for industrial processes.

In our lab, we focus on a class of enzymes called Fructosyl Amino Acid Oxidases (FAOX), which are flavoproteins that catalyze the oxidation of fructosyl amino acids to form glucosone, amino acid and hydrogen peroxide which has major role in the management of diabetes, and specifically in the detection of glycated hemoglobin (HbA1c).

However, naturally occurring FPOX are not able to detect HbA1c directly because these enzymes show no significant activity on intact proteins due to the buried active site and to the narrow tunnel that provides access to their catalytic pocket, depicted by the crystal structures of FAOX and FPOX enzymes. Hence, the need to expand their substrate range by enzyme engineering.

We applied a rational design approach to engineer a novel enzyme with a wider access tunnel to the catalytic site, using a combination of Rosetta design and molecular dynamics simulations.

We have been successful in designing several mutants shows a significantly wider and shorter access tunnel, relative to the wild-type (WT) enzyme. Upon experimental testing, engineered enzyme shows good structural stability and maintains significant activity relative to the WT. Also, the thermal stability of WT enzyme has been improved with the variants that have increased salt bridges, improved RMSF, improved native contacts, and disulphide bonds. We are determined the structures by X-Ray Crystallography of the engineered enzyme, studied its biophysical properties and determined its activity

We are currently working to test the enzymes on glycated proteins and to produce nanofibers embedding them in a biomaterial for diagnostic application.

References:

[1] Gautieri A, Rigoldi F, Torretta A, Redaelli A, Parisini E. In silico engineering of enzyme access tunnels. In: “Methods in Molecular Biology” (2021).

[2] Rigoldi F, Donini S, Torretta A Parisini E, Gautieri A Biotechnol. Bioeng. 2020, 117, 3688.

P 6. Artificial metalloproteins based on the Spy technology: molecular modelling and catalysis.

Valentina Borghesani[1], Chiara Bottoni[1], Irene Borghesi[1], Alessandro Nicoli[2], Antonella Di Pizio[2], Matteo Tegoni[1]

valentina.borghesani@unipr.it

[1] Department of Chemistry, Life Sciences, and Environmental Sustainability, University of Parma, 43123 Parma, Italy
[2] Leibniz Institute for Food Systems Biology at the Technical University of Munich, 85354 Freising, Germany

Metalloproteins promote several of the most complex biomolecular processes in nature. The design of new metalloproteins is therefore of interest in the field of the development of new efficient biocatalysts which can carry out reactions that are not relevant for biological systems but are important for applications in bio- and nanotechnology[1]. In the redesign of metalloproteins one of the major challenges is the introduction of metal binding sites in specific position of the construct, here makes possible by the "Trojan-horse" strategy. A peptide, bearing a metal site, interacts with a protein anchoring a metal site to the protein.

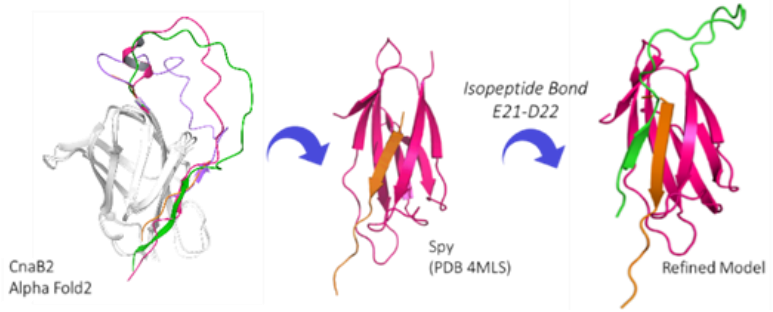


Figure 1. Representation of the molecular modeling approach used to refine the SpyCatcher/SpyTag complex. A refined models of CnaB2 (PDB: 2X5P) with the predicted N-terminus β-strand loop was used for Spy complex (PDB: 4MLS) refinement and isopeptide bond parametrization. The resulting SpyCatcher/SpyTag refined model shows in green the predicted N-terminal β-strand (SpyCatcher. Magenta; SpyTag. Orange).

Here we present a molecular modeling study of the SpyCatcher/SpyTag complex. The SpyCatcher construct is a β-barrel protein (rational optimized by Howarth and co-workers2 starting from the domain CnaB2). SpyCatcher (Figure 1, magenta) binds covalently an oligopeptide called SpyTag (Figure 1, orange) through the formation of an isopeptide bond between an Asp and a Lys residues. However, since the crystallography structure of CnaB2 it is not completely resolved also the Spy complex is not completely resolved in the N-terminal region. By a complementary approach between homology modeling and artificial intelligence modeling we demonstrate that Spy's predicted structure could complete with a N-terminus β-strand loop (Figure 1, green). This computational study will have a great impact for future metal enzyme design development since it will allow us to better understand the second sphere coordination and herein became able to modify it in order to modulate the catalytic activity of our systems.

We also have fully characterized the SpyTag ad SpyCatcher independently, as well as the SpyCatcher/SpyTag construct, by absorption, CD and fluorescence, proving the binding of metal ions occurs sequentially in the Spy complex.

Finally, we are here going to discuss also the catalytic properties of Spy complexes in the oxidation of catechols, and how we can enhance this catalytic activity and/or stereoselectivity.

Acknowledgements:

Project "Artificial enzymes for the photocatalytic production of hydrogen in photosynthetic bacteria" funded under the National Recovery and Resilience Plan (NRRP), Mission 2 Component 2 Investment 3.5 - Call for tender No. 4 of March 23, 2022 of Italian Ministry of Ecologic Transition funded by the European Union – NextGenerationEU. Project code RSH2A_000009, Concession Decree 445 of December 29, 2022 adopted by the Italian Ministry of Environment and Energy Security, CUP F97G22000270006. This work has benefited from the equipment and framework of the COMP-HUB and COMP-R Initiatives, funded by the 'Departments of Excellence' program of the Italian Ministry for University and Research (MIUR, 2018-2022 and MUR, 2023-2027).

References:

[1] F. Yu, V.M. Cangelosi, M.L. Zastrow, M. Tegoni, J.S. Plegaria, A.G. Tebo, C.S. Mocny, L. Ruckthong, H. Qayyum, V.L. Pecoraro, Protein Design: Toward Functional Metalloenzymes. Chemical Reviews 2014, 114 (7), 3495-3578.

[2] S.C. Reddington, M. Howarth, Secrets of a covalent interaction for biomaterials and biotechnology: SpyTag and SpyCatcher. Current Opinion in Chemical Biology 2015, 29, 94-99.

P 7. Biocatalyst for the synthesis of D-amino-acids: fine-tuning the immobilization of engineered amino acid dehydrogenase

Krisztina Boros[1], Lilla Cserepes[1,2], Raluca Bianca Tomoiagă[1], Anca Anghel[1], Jörg Pietruszka[2], László Csaba Bencze[1]

krisztina.boros@ubbcluj.ro

[1] Enzymology and Applied Biocatalysis Research Centre, Babeș-Bolyai University, Arany János str. 11, RO-400028 Cluj-Napoca, Romania

[2] Institute of Bio- and Geosciences, Biotechnology, Forschungszentrum Jülich GmbH, Wilhelm-Johnen str., DE-52428, Jülich, Germany

Aromatic D-amino acids (D-AAs) have gained increasing attention in both industrial and pharmaceutical fields.[1] Biocatalytic procedures for the synthesis of D-AAs with high enantiopurity emerged as environmentally friendly alternatives of high productivity.[2]

Recently, the thermostable *meso*-2,6-diamino-pimelic acid D-dehydrogenase (DAPDH) from *Ureibacillus thermosphaericus*, transformed *via* protein engineering into D-selective amino acid dehydrogenase (DAADH) was employed to catalyze the reductive amination of different phenylpyruvic acids into D-phenylalanines of high synthetic interest.[3] Since *Ut*DAADH requires the presence of the reduced form of expensive NADPH, the cofactor's regeneration by auxiliary enzymes, such as glucose dehydrogenase, is mandatory for a cost-efficient bioprocess (Fig. 1).

Furthermore, enzyme immobilization provides robust biocatalysts with high operational stability and recyclability enabling the enhancement of productivity and cost-effectiveness within the bioprocess. Thus, in our work particular emphasis was placed on the immobilization of the engineered *Ut*DAADH, involving comprehensive evaluation of various commercially accessible and in-house developed support materials and linkers, as well as different types of binding approaches, such as adsorption-based and covalent non-specific and site-specific immobilization methods.

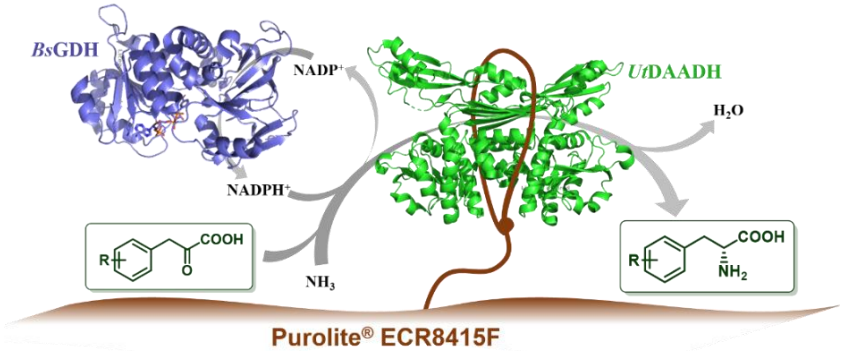


Figure 1. Reductive amination of phenylpyruvic acids into D-phenylalanines, catalyzed by immobilized *Ut*DAADH, coupled with *Bacillus subtilis* glucose dehydrogenase (*Bs*GDH) mediated cofactor regeneration.

Among the several assessed procedures, covalent immobilization onto Purolite ECR8415F resin proved to be the most efficient and economically viable. Consequently, this approach underwent further refinement at numerous levels. With the optimized procedure established, site-specific immobilization of the DAADH enzyme was attempted on the appropriately

functionalized Purolite resin, employing maleimide-thiol coupling.[4] Surface-exposed serine residues, positioned far from the active site, were selected and successfully mutated to cysteines to enable this linkage. The catalytic efficiency and recyclability of the obtained biocatalysts were evaluated, to obtain efficient and highly recyclable immobilized biocatalysts, suitable for the cost-effective production of D-phenylalanine analogues.

References:

[1] S. Martínez-Rodríguez, A.I. Martínez-Gómez, F. Rodríguez-Vico, J.M. Clemente-Jiménez, F.J. Las Heras-Vázquez. Natural Occurrence and Industrial Applications of D-Amino Acids: An Overview. Chemistry and Biodiversity 2010, 7 (6); 1531-1548.

[2] a) S.D. Tork, E.Z.A. Nagy, L. Cserepes, D.M. Bordea, B. Nagy, M.I. Toşa, C. Paizs, L.C. Bencze. The production of L- and D-phenylalanines using engineered phenylalanine ammonia lyases from Petroselinum crispum. Scientific Reports 2019, 9 (1); 20123. b) E. Heuson, F. Charmantray, J.L. Petit, V. Berardinis, T. Gefflaut. Enantioselective Synthesis of D- and L-α-Amino Acids by Enzymatic Transamination Using Glutamine as Smart Amine Donor. Advanced Synthesis and Catalysis 2019, 361 (4); 778-785. c) F. Parmeggiani, S.T. Ahmed, M.P. Thompson, N.J. Weise, J.L. Galman, D. Gahloth, M.S. Dunstan, D. Leys, N.J. Turner. Single-Biocatalyst Synthesis of Enantiopure D-Arylalanines Exploiting an Engineered D-Amino Acid Dehydrogenase. Advanced Synthesis and Catalysis 2016, 358 (20); 3298-3306.

[3] H. Akita, K. Doi, Y. Kawarabayasi, T. Ohshima. Creation of a thermostable NADP+-dependent D-amino acid dehydrogenase from Ureibacillus thermosphaericus strain A1 meso-diaminopimelate dehydrogenase by site-directed mutagenesis. Biotechnology Letters 2012, 34; 1693-1699.

[4] K. Boros, M.E. Moisă, C.L. Nagy, C. Paizs, M.I. Toşa, L.C. Bencze. Robust, site-specifically immobilized phenylalanine ammonia-lyases for the enantioselective ammonia addition of cinnamic acids. Catalysis Science and Technology 2021, 11 (16); 5553-5563.

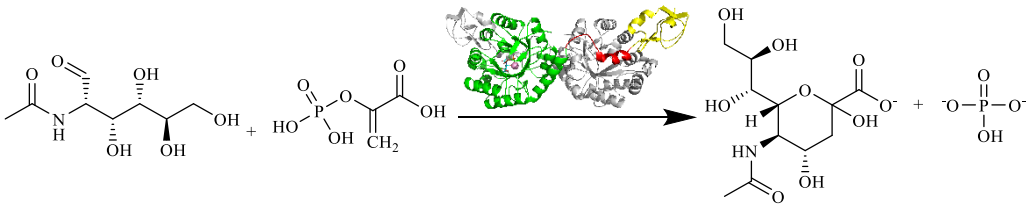
P 8. Model-based Optimization of Neu5Ac Synthesis

Mehmet Mervan Çakar[1], Theofania Andreadaki[2], Simon Charnock[2], Wolf-Dieter Fessner[3], Zvjezdana Findrik Blažević[1]

mcakar@fkit.hr

[1] Faculty of Chemical Engineering and Technology, University of Zagreb, Savska c. 16, 10000, Zagreb, Croatia
[2] Prozomix Limited, Station Court, Haltwhistle, Northumberland, NE49 9HN, UK
[3] Institute of Organic Chemistry and Biochemistry, Technische Universitaet Darmstadt, Alarich-Weiss-Strasse 4, 64287 Darmstadt, Germany.

Sialic acids (SAs) are a group of more than 50 structurally distinct α-keto acids found in viruses, mammalian cells, and microorganisms.[1] They control various biological functions such as development, recognition, cell signaling, cell-cell interactions, and adhesion.[2] Sialylation of neural cell adhesion molecules during embryonic development is crucial for proper neural tissue development, while in certain cancers, sialylation is correlated with tumorigenesis and metastasis.[3] Pathogenic bacteria use sialylated glycoproteins and glycolipids to mask their presence from the host's immune system. There are over fifty molecules of sialic acid that carry diverse substituents at hydroxyl or amino groups, and their distribution is strongly regulated on a gene level and varies depending on the species of animal and cell's function.[4] *N*-Acetylneuraminic acid (Neu5Ac) is the most commonly occurring and studied SA, and its biosynthesis is controlled by Neu5Ac synthase, which catalyzes the aldol-like condensation of phosphoenolpyruvate (PEP) to N-Acetylmannosamine (ManNAc) to yield Neu5Ac.



Scheme 1. NeuS catalyzed Neu5Ac synthesis from ManNAc and PEP via aldol-like addition

In this work, a thorough kinetic analysis of Neu5Ac synthase (NeuB) catalysed synthesis of Neu5Ac was done in order to build the kinetic model and use it to find the bottlenecks of the system and optimize the reaction. The interdependent relationships between process variables of NeuB from *Neisseria meningitidis* and a promising NeuB homologue from metagenomic library were successfully investigated and evaluated for their potential use in SA synthesis.[5]

Acknowledgements:

This project has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 956631.

References:

[1] A. Varki. Biological roles of oligosaccharides: All of the theories are correct. Glycobiology 1993, 3, 97–130.

[2] B. A. Cunningham, S. Hoffman, U. Rutishauser, J. J. Hemperly, G. M. Edelman. Molecular topography of the neural cell adhesion molecule N-CAM: Surface orientation and location of sialic acid-rich and binding regions. Proceedings of the National Academy of Sciences of the United States of America 1983, 80, 3116–3120.

[3] E. Gorelik, U. Galili, A. Raz. On the role of cell surface carbohydrates and their binding proteins (lectins) in tumor metastasis. Cancer and Metastasis Reviews 2001, 20, 245–277.

[4] R. Schauer. Biochemistry of sialic acid diversity. Carbohydrates in Chemistry and Biology 2008, 3–4, 227–243.

[5] M. Sudar, Z. F. Blažević. Enzyme Cascade Kinetic Modelling. in Enzyme Cascade Design and Modelling (eds. Kara, S. & Rudroff, F.). 2021, 91–108.

P 9. Enzyme engineering studies of rat mitochondrial 2E-enoyl-CoA hydratase (ECH) aimed at changing its substrate specificity for using it in synthetic biology applications

Subhadra Dalwani[1], Olga Pereshivko[2], Vsevolod Peshkov[2], Eeki Tarkkonen[2], Werner Schmitz[3], Petri M. Pihko[2], Rik K. Wierenga[1]

[1] Faculty of Biochemistry and Molecular Medicine, University of Oulu
[2] Department of Chemistry and Nanoscience Center, University of Jyväskylä
[3] Theoder-Boveri-Institut für Biowissenschaften der Universität Würzburg, Germany

The Crotonase Superfamily (CS) is a well-studied mechanistically diverse superfamily of enzymes. Its members possess a conserved canonical fold (the crotonase fold) and its members have an active site oxyanion hole formed by two main chain amide nitrogen atoms, which provides the chemical capability of stabilizing oxyanion/enolate intermediates formed during catalysis. Despite these conserved features, members of the CS can have as little as 10% sequence identity and are known to catalyze varied reactions, which makes these enzymes interesting targets for use in bio-catalytic based biosynthetic/biotechnological applications. At least 32 CS enzymes of known function have been characterized, some having the ability to catalyze more than one reaction. The CoA dependent enoyl-CoA hydratase catalyzes the enantio- and diasteroselective addition of water to 2*E*-enoyl-CoA molecules producing chirally pure 3S-hydroaxyacyl-CoA molecules. Such an enantioselective addition of water is very challenging to carry out by standard organic-chemistry methods. Additionally, the enzyme is capable of catalyzing the hydration of crotonyl-pantetheine in the presence of either adenosine 3'5' diphosphate or Coenzyme A, indicating that these can be used as activators for enzyme catalysis. These features make ECH an attractive target for enzyme engineering projects. In our project, we aim to change the substrate specificity of ECH, by using as substrates truncated variants of CoA, such as the PAN and NAC moieties and by changing the acyl moiety such that the hydrated product can be used as an intermediate in further synthetic efforts. Initially, efforts will be directed towards RnECH1 and the binding properties of this enzyme with truncated acyl-NAC and acyl-PAN compounds (provided by the research group of Prof. Petri Pihko at the University of Jyväskylä) will be characterized using methods such as direct end point and continuous kinetic activity assays with Mass Spectrometry, Spectrophotometric kinetic assays, Isothermal Titration Calorimetry, Crystallographic binding studies and Biocomputational approaches. The effect of different activators on the binding properties will also be tested. Based on the insight gained from these experiments, more NAC and PAN compounds with acyl tails having various functional groups will be synthesized and tested to optimize the system for the syntheses of relevant β-hydroxythioesters, α-methyl-β-hydroxythioesters and other thioesters.

P 10. Insights into the structural determinants of substrate specificity and catalysis of bacterial C-glycoside oxidases

Tomás Frazão[1], Lígia O. Martins[1]

t.frazao@itqb.unl.pt

[1] Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa, Av da República, 2780-157 Oeiras, Portugal

C-glycosides are natural sugar-containing molecules that are abundant in plants (flowers and fruits), including dietary ones, and are more resistant to degradation than O-, N- and S-glycosides, hindering the availability of their sugar and bioactive aglycone motif.[1] Glycoside 3-oxidases (G3Oxs), are newly identified bacterial flavo-oxidases from the glucose-methanol-choline (GMC) superfamily that catalyze the oxidation of C-glycosides with the concomitant reduction of O_2 to H_2O_2. This oxidation of the sugar motif is followed by a C-C metal-assisted acid/base bond cleavage carried out by C-deglycosylases (CDGs), releasing the sugar and free aglycone in two-step C-deglycosylation pathways.[2] Soil and gut microorganisms have different oxidative enzymes responsible for the catabolic pathways of these compounds, but the details of their catalytic mechanisms are largely unknown. In our work, we report that the bacterial *Ps*GO3x, previously thought of as a pyranose oxidase (*Ps*P2Ox) [3], oxidizes the glucose moiety of the C-glycoside mangiferin, producing 3-keto-mangiferin, at 50,000-fold higher specificity (kcat/Km) than free D-glucose. Four proteins similar to known CDG complexes can be encoded close to the *Ps*G3Ox gene, composing a putative C-deglycosylation catabolic pathway. Analysis of *Ps*G3Ox, *Ps*G3Ox-Glucose, and *Ps*G3Ox-Mangiferin X-ray crystal structures, combined with mutagenesis, molecular dockings, and molecular dynamics, revealed that a substrate loop located at the active site entrance, and an insertion loop, not found in pyranose oxidases, act together as dynamic modulators of the substrate access and accommodation in the active site, as well as substrate regioselectivity. Key non-catalytic residues responsible for substrate stabilization and selectivity were unveiled. This work revealed important structure-to-function relationships among this family of enzymes and was crucial to expand our knowledge on G3Oxs and their differences from P2Oxs, namely their substrate specificity.

References:

[1] Xiao J, Capanoglu E, Jassbi AR, Miron A. Advance on the Flavonoid C-glycosides and Health Benefits. Crit Rev Food Sci Nutr. 2016 Jul 29, 56:S, 29–45.

[2] Mori T, Kumano T, He H, Watanabe S, Senda M, Moriya T, et al. C-Glycoside metabolism in the gut and in nature: Identification, characterization, structural analyses and distribution of C-C bond-cleaving enzymes. Nat Commun. 2021 Dec 1, 12(1).

[3] Mendes S, Banha C, Madeira J, Santos D, Miranda V, Manzanera M, et al. Characterization of a bacterial pyranose 2-oxidase from Arthrobacter siccitolerans. J Mol Catal B Enzym. 2016 Aug 3, 133:S, 34–43.

P 11. Expanding the Substrate Scope of Fluoroacetate Dehalogenase Enzymes via Directed Evolution

Amy Gooch

amy.gooch@oist.jp

Protein Engineering and Evolution Unit, Okinawa Institute of Science and Technology

Fluoroacetate dehalogenases (FAcDs) are unique enzymes capable of cleaving the strongest covalent bond in organic chemistry: that between carbon and fluorine (C-F).[1] However, FAcDs remain limited in their ability to defluorinate more highly fluorinated compounds, and therefore in their utility in applications such as the bioremediation of fluorinated pollutants.[2] To address these limitations, we aim to evolve FAcD substrate scope towards more highly fluorinated compounds via directed evolution (DE). To achieve this aim, we first explored the natural functional diversity of FAcD enzymes via biochemical characterization of 20 homologs selected from a sequence similarity network (SSN). Protein expression analysis and activity screening via ^{19}F NMR revealed 14 soluble proteins exhibiting defluorinase activity. Additional kinetic assays showed varying rates of defluorination, including activity on difluorinated and less-activated substrate analogs. These results not only yield insight into the structural features influencing substrate acceptance and preference of these enzymes, but also provide promising starting points for DE. In particular, FAcD 9 stood out as a good starting point for DE due to its favorable properties, including its relatively efficient expression, broad substrate promiscuity and reasonable stability. To gain insight into the active site environment of FAcD 9, we next undertook X-ray crystallography experiments. We solved the X-ray crystal structure of apo FAcD 9 at 1.2 Å resolution and the enzyme−substrate covalent intermediate at 1.3 Å resolution. This high-resolution data revealed the detailed structure of the active site and will be used to inform the design of targeted mutations in FAcD 9. Specifically, we aim to use this structural data in combination with computational tools in order to engineer FAcD activity and produce smart mutant libraries for DE. Altogether, these results provide the basis to evolve FAcD substrate scope towards more highly fluorinated compounds, an aim that will significantly improve the prospect of applying these enzymes to the bioremediation of toxic and environmentally persistent fluorinated pollutants.

References:

[1] P. W. Y. Chan, A. F. Yakunin, E. A. Edwards, E. F. Pai. Mapping the Reaction Coordinates of Enzymatic Defluorination. Journal of the American Chemical Society 2011, 133 (19), 7461-7468.

[2] L. P. Wackett. Strategies for the Biodegradation of Polyfluorinated Compounds. Microorganisms 2022, 10 (8), 1664-1676.

P 12. Enhancing the stability of PET-degrading DmPETase with rational and computational enzyme design

Konstantinos Grigorakis[1], Sergio M. Marques[2,3], Konstantinos Makryniotis[1], Efstratios Nikolaivits[1], David Bednar[2], Evangelos Topakas[1]*

***vtopakas@central.ntua.gr**

[1] Industrial Biotechnology & Biocatalysis Group, Biotechnology Laboratory, School of Chemical Engineering, National Technical University of Athens, Athens, Greece
[2] Loschmidt Laboratories, Department of Experimental Biology and RECETOX, Faculty of Science, Masaryk University, Kamenice 5, 625 00 Brno, Czech Republic
[3] International Clinical Research Center, St. Anne's University Hospital Brno, Pekarska 53, 656 91 Brno, Czech Republic

Polyethylene terephthalate (PET) is the most recyclable and recycled plastic worldwide, the most widely used material for beverage packaging and valuable enough to solicit R&D projects to its after-use recovery.[1] Enzymatic depolymerization of PET to its smaller components, terephthalic acid (TPA) and ethylene glycol (EG) allows for its separation from plastic mixtures but also enables production of high quality repolymerized PET. This is in contrast to material-degrading thermomechanical recycling methods.[2] Several reported PET-hydrolase (PETase) enzymes enable this waste−PET biorefinery but require protein stability and activity engineering to achieve industrial viability.[3] Recently characterized thermophilic DmPETase, a PETase that is less affected by PET crystallinity increase than the benchmark PETase LCC^{ICCG}, serves as a new template for engineering more efficient PET−depolymerizing enzymes.[4] In this study, we attempted to engineer the stability of DmPETase using three different approaches: 1) designing disulfide bridges, 2) designing single point mutations with automated pipelines, and 3) ancestral sequence reconstruction. Two 200 ns MD simulations of a DmPETase homology model, prepared using SWISS-MODEL,[5] were performed with Amber[6] and used to predict the flexibility of each residue. Several snapshots from those MDs were used with Disulfide by Design 2.0,[7] which generated a list of potential disulfide bridges. PROSS[8] and FireProt 2.0[9] were used to predict point mutations, using models created with SWISS-MODEL and AlphaFold 2.0.[10] To select among the predicted stabilizing mutations, we compared them directly with the available literature for engineered variants of IsPETase and LCC, and also used machine learning-based MūtCompute[11] predictions for IsPETase and LCC. Point mutations were verified using PyMOL for mutagenesis and visual inspection, as well as Rosetta12 for ΔΔG prediction of single point mutations. Ancestral sequence reconstruction was performed using FireProt-ASR13 to predict potentially stable ancestral enzymes. Engineered mutations beneficial to DmPETase stability could help design better PET−depolymerizing biocatalysts.

Acknowledgements:

This poster is based upon work from COST Action CA21162 COZYME, supported by COST (European Cooperation in Science and Technology).

References:

[1] Sarda, P., Hanan, J. C., Lawrence, J. G., Allahkarami, M. Sustainability Performance of Polyethylene Terephthalate, Clarifying Challenges and Opportunities. Journal of Polymer Science 2022, 60 (1), 7–31. https://doi.org/10.1002/POL.20210495.

[2] Kaabel, S., Daniel Therien, J. P., Deschênes, C. E., Duncan, D., Friščic, T., Auclair, K. Enzymatic Depolymerization of Highly Crystalline Polyethylene Terephthalate Enabled in Moist-Solid Reaction Mixtures. Proc Natl Acad Sci U S A 2021, 118 (29), e2026452118. https://doi.org/10.1073/PNAS.2026452118/SUPPL_FILE/PNAS.2026452118.SAPP.PDF.

[3] Tournier, V., Topham, C. M., Gilles, A., David, B., Folgoas, C., Moya-Leclair, E., Kamionka, E., Desrousseaux, M. L., Texier, H., Gavalda, S., Cot, M., Guémard, E., Dalibey, M., Nomme, J., Cioci, G., Barbe, S., Chateau, M., André, I., Duquesne, S., Marty, A. An Engineered PET Depolymerase to Break down and Recycle Plastic Bottles. Nature 2020 580:7802 2020, 580 (7802), 216–219. https://doi.org/10.1038/s41586-020-2149-4.

[4] Makryniotis, K., Nikolaivits, E., Gkountela, C., Vouyiouka, S., Topakas, E. Discovery of a Polyesterase from Deinococcus Maricopensis and Comparison to the Benchmark LCC ICCG Suggests High Potential for Semi-Crystalline Post-Consumer PET Degradation. J Hazard Mater 2023, 455, 131574. https://doi.org/10.1016/j.jhazmat.2023.131574.

[5] Waterhouse, A., Bertoni, M., Bienert, S., Studer, G., Tauriello, G., Gumienny, R., Heer, F. T., De Beer, T. A. P., Rempfer, C., Bordoli, L., Lepore, R., Schwede, T. SWISS-MODEL: Homology Modelling of Protein Structures and Complexes. Nucleic Acids Res 2018, 46 (W1), W296–W303. https://doi.org/10.1093/NAR/GKY427.

[6] Case, D. A., Aktulga, H. M., Belfon, K., Ben-Shalom, I. Y., Berryman, J. T., Brozell, S. R., Cerutti, D. S., Cheatham, T. E. , I., Cisneros, G. A., Cruzeiro, V. W. D., Darden, T. A., Forouzesh, N., Giambaşu, G., Giese, T., Gilson, M. K., Gohlke, H., Goetz, A. W., Harris, J., Izadi, S., Izmailov, S. A., Kasavajhala, K., Kaymak, M. C., King, E., Kovalenko, A., Kurtzman, T., Lee, T. S., Li, P., Lin, C., Liu, J., Luchko, T., Luo, R., Machado, M., Man, V., Manathunga, M. K. M., Miao, Y., Mikhailovskii, O., Monard, G., Nguyen, H., K.A. O'Hearn, Onufriev A., Pan F., Pantano S., Qi R., Rahnamoun A., Roe D.R., Roitberg A., Sagui C., Schott-Verdugo S., Shajan A., Shen J., Simmerling C.L., Skrynnikov N.R., Smith J., Swails J., Walker R.C., Wang J., Wang J., Wei H., Wu X., Wu Y., Xiong Y., Xue Y., York D.M., Zhao S., Zhu Q., Kollman P.A. Amber 2023 Reference Manual; University of California: San Francisco , 2023.

[7] Craig, D. B., Dombkowski, A. A. Disulfide by Design 2.0: A Web-Based Tool for Disulfide Engineering in Proteins. BMC Bioinformatics 2013, 14 (1), 346. https://doi.org/10.1186/1471-2105-14-346.

[8] Goldenzweig, A., Goldsmith, M., Hill, S. E., Gertman, O., Laurino, P., Ashani, Y., Dym, O., Unger, T., Albeck, S., Prilusky, J., Lieberman, R. L., Aharoni, A., Silman, I., Sussman, J. L., Tawfik, D. S., Fleishman, S. J. Automated Structure- and Sequence-Based Design of Proteins for High Bacterial Expression and Stability. Mol Cell 2016, 63 (2), 337–346. https://doi.org/10.1016/j.molcel.2016.06.012.

[9] Musil, M., Stourac, J., Bendl, J., Brezovsky, J., Prokop, Z., Zendulka, J., Martinek, T., Bednar, D., Damborsky, J. FireProt: Web Server for Automated Design of Thermostable Proteins. Nucleic Acids Res 2017, 45 (W1), W393–W399. https://doi.org/10.1093/nar/gkx285.

[10] L Jumper, J., Evans, R., Pritzel, A., Green, T., Figurnov, M., Ronneberger, O., Tunyasuvunakool, K., Bates, R., Žídek, A., Potapenko, A., Bridgland, A., Meyer, C., Kohl, S. A. A., Ballard, A. J., Cowie, A., Romera-Paredes, B., Nikolov, S., Jain, R., Adler, J., Back, T., Petersen, S., Reiman, D., Clancy, E., Zielinski, M., Steinegger, M., Pacholska, M., Berghammer, T., Bodenstein, S., Silver, D., Vinyals, O., Senior, A. W., Kavukcuoglu, K., Kohli, P., Hassabis, D. Highly Accurate Protein Structure Prediction with AlphaFold. Nature 2021 596:7873 2021, 596 (7873), 583–589. https://doi.org/10.1038/s41586-021-03819-2

[11] Lu, H., Diaz, D. J., Czarnecki, N. J., Zhu, C., Kim, W., Shroff, R., Acosta, D. J., Alexander, B. R., Cole, H. O., Zhang, Y., Lynd, N. A., Ellington, A. D., Alper, H. S. Machine Learning-Aided Engineering of Hydrolases for PET Depolymerization. Nature 2022, 604 (7907), 662–667. https://doi.org/10.1038/s41586-022-04599-z.

[12] Kellogg, E. H., Leaver-Fay, A., Baker, D. Role of Conformational Sampling in Computing Mutation-Induced Changes in Protein Structure and Stability. Proteins: Structure, Function, and Bioinformatics 2011, 79 (3), 830–838. https://doi.org/10.1002/prot.22921.

[13] Musil, M., Khan, R. T., Beier, A., Stourac, J., Konegger, H., Damborsky, J., Bednar, D. FireProtASR: A Web Server for Fully Automated Ancestral Sequence Reconstruction. Brief Bioinform 2021, 22 (4). https://doi.org/10.1093/bib/bbaa337.

P 13. GASP: A pan-specific predictor of family 1 glycosyltransferase specificity enabled by a pipeline for substrate feature generation and large-scale experimental screening

David Harding-Larsen[1,§], Christian D. Madsen[1,§], David Teze[1], Tiia Kittilä[1], Mads Rosander Langhorn[1], Hani Gharabli[1], Mandy Hobusch[1], Felipe Mejia Otalvaro[1], Onur Kırtel[1], Stanislav Mazurenko[2], Evelyn Travnik[1], Ditte H. Welner[1]

dahala@dtu.dk

[§]Contributed equally to this work.
1 Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, 2800 Kongens Lyngby, Denmark
2 Loschmidt Laboratories, Department of Experimental Biology and RECETOX, Faculty of Science, Masaryk University, Kotlarska 2, Brno 61137, Czech Republic

Enzymes hold great potential in various domains, including sustainable industrial processes for chemical, energy, and materials production. One of these domains is glycosylation, a crucial step to obtaining a plethora of biologically and industrially relevant molecules, from proteins to natural products and artificial compounds.[1] In nature, these reactions are mainly catalysed by glycosyltransferases, enzymes which offer perfect stereoselectivity and often high regioselectivity in a single reaction with unprotected substrates.[2] However, comprehending the intricate catalytic mechanisms of these proteins are often challenging due to their vast acceptor specificity. While the significance of protein structure and sequence in determining activity and function is well-known, predicting protein behavior solely from these factors remains difficult. Here, Machine Learning offers a promising avenue for addressing this issue by uncovering hidden patterns and relationships from the available data.[3]

In this study, we present the Glucosyltransferase Acceptor Specificity Predictor (GASP) model, a data-driven approach to the identification of reactive GT1:acceptor pairs. We trained a random forest-based acceptor predictor on literature data and validated it on independent in-house generated data on 1001 GT1:acceptor pairs, obtaining an AUROC of 0.79 and a balanced accuracy of 72%. GASP is capable of automatically parsing all known GT1 sequences, as well as all substrates, the latter through a pipeline for the generation of 153 chemical features for any given molecule taking the CID as input. The predictor had a 50% hit rate in a comparative case study for the glycosylation of the plant defensive compound DIBOA, compared to an 83% hit rate with expert-selected enzymes. Furthermore, using Explainable AI [4] approaches such as negative feature selection, we identified properties related to cyclization and atom hybridization status to be the most important characteristics for accurate prediction.

In conclusion, this study introduces a ready-to-use predictor for GT1:acceptor pairs. Not only does the automatic nature of the model enable future predictors to be trained using new data, the modular architecture of the pipelines also allows for the incorporation of new protein representations..

References:

[1] B. Nidetzky et al. Glycosyltransferases as Biocatalysts for Chemical Production. ACS Catal 2018, 8 (7), 6283–6300.

[2] D. Bowles et al. Glycosyltransferases: Managers of Small Molecules. Curr Opin Plant Biol 2005, 8 (3), 254–263.

[3] R. Bonetta, G. Valentino. Machine learning techniques for protein function prediction. Proteins 2019, 88 (3), 387-413.

[4] G. P. Wellawatte, H. A. Gandhi, A. Seshadri, A. D. White. A Perspective on Explanations of Molecular Prediction Models. J. Chem. Theory Comput. 2023, 19 (8), 2149–2160.

.

P 14. The role of conformational dynamics in the evolution of designed Kemp eliminase

Gyula Hoffka[1,2], Shina Caroline Lynn Kamerlin[3,4]

[1] Laboratory of Retroviral Biochemistry, Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of Debrecen, Hungary
[2] Doctoral School of Molecular Cell and Immune Biology, University of Debrecen, Debrecen, Hungary
[3] Department of Chemistry – BMC, Uppsala University, Uppsala, Sweden
[4] School of Chemistry and Biochemistry, Georgia Institute of Technology, Atlanta, Georgia, USA

Enzymes are able to enhance reaction rates by several orders of magnitude. Recent studies confirm the role of conformation heterogeneity in the evolution of novel activities. The role of distant mutations in this regard is still unclear. Computational methods allow us to examine properties of enzymes that would prove to be challenging with experimental methods. The state-of-the-art Empirical Valence Bond (EVB) method is able to reproduce observed activation energies with exceptionally high accuracy, enabling us to carry out in-depth theoretical studies on enzyme evolution.

The Kemp elimination reaction is frequently studied in the context of artificial evolution, with several previous and ongoing attempts at creating an effective catalyst of the reaction. One of the most successful Kemp eliminase, HG-3.17 was derived using a combined computational and experimental approach, inserting a designed active site into a TAX xylanase scaffold and applying directed evolution to increase the activity. The application of EVB to study the evolution leading to HG-3.17 provides us with an excellent opportunity to examine the molecular background of evolution, and provide us with a deeper understanding of conformational selection in the optimization of enzyme activity.

P 15. Shedding Light on the Secrets of NanoLuc, Its Mechanism, and Allosteric Behaviour

Jana Horáčková[1], Michal Nemergut[1,2], Daniel Pluskal[1], Tereza Šustrová[1], Jan Tulis[1], Tomáš Bárta[3], Racha Baatallah[4], Glwadys Gagnot[4,5], Veronika Nováková[1,2], Marika Majerová[1,2], Karolína Sedláčková[1,2], Sérgio M. Marques[1,2], Martin Toul[1,2], Jiří Damborský[1,2], Zbyněk Prokop[1,2], Yves L. Janin[6], Martin Marek[1,2], David Bednář[1,2]

jana.horackova@recetox.muni.cz

[1] Loschmidt Laboratories, Department of Experimental Biology and RECETOX, Faculty of Science, Masaryk University, Kamenice 5, Bld. C13, 625 00 Brno, Czech Republic
[2] International Clinical Research Center, St. Anne's University Hospital Brno, Pekarska 53, 656 91 Brno, Czech Republic
[3] Department of Histology and Embryology, Faculty of Medicine, Masaryk University, Kamenice 753/5, 625 00 Brno, Czech Republic
[4] Unité de Chimie et Biocatalyse, Institut Pasteur, UMR 3523, CNRS, 28 rue du Dr. Roux, 75724 Paris Cedex 15, France
[5] Université de Paris, 12 rue de l'école de Médecine, 75006 Paris, France
[6] Structure et Instabilité des Génomes (StrInG), Muséum National d'Histoire Naturelle, INSERM, CNRS, Alliance Sorbonne Université, 75005 Paris, France

NanoLuc is a small but exceptionally bright luciferase enzyme that is widely used in biotechnology and biomedicine. It was designed in 2012 by mutating the luciferase of a deep-sea shrimp *Oplophorus gracilirostris*.[1] However, the mechanism of NanoLuc's light-emitting reaction, which is vital for the successful development of next-generation bioluminescent systems, has not been solved. Therefore, we applied multiple lab- and computer-based methods to thoroughly study NanoLuc and its catalysis, including crystallography, kinetic measurements, molecular docking, and molecular dynamics simulations with enhanced sampling.

One of the advantages of NanoLuc is its small size of 171 amino acid residues compared to luciferases from sea pansy *Renilla reniformis* (311 residues) and firefly Photinus pyralis (550 residues). We confirmed that NanoLuc is indeed monomeric in solution, however, in some crystals, it is packed as a crystallographic homotetramer. Moreover, we identified two different binding sites of the substrate molecule: the catalytic site which is buried in the core of NanoLuc in its monomeric form, and an allosteric binding pocket shaped on the oligomerization interface of NanoLuc crystals2. Interestingly, we observed that some single-point NanoLuc mutants, where we restored the sequence of the *Oplophorus* luciferase, exist in the form of a monomer-tetramer mixture. Therefore, we hypothesize that the tetrameric form of *Oplophorus* luciferase featuring a substrate molecule bound to the allosteric site serves as a reservoir to produce light upon changes in external conditions.

References:

[1] M.P. Hall, J. Unch, B.F. Binkowski, M.P. Valley, B.L. Butler, M.G. Wood, P. Otto, K. Zimmerman, G. Vidugiris, T. Machleidt, M.B. Robers, H.A. Benink, C.T. Eggers, M.R. Slater, P.L. Meisenheimer, D.H. Klaubert, F. Fan, L.P. Encell, K.V. Wood. Engineered Luciferase Reporter from a Deep Sea Shrimp Utilizing a Novel Imidazopyrazinone Substrate. ACS Chemical Biology 2012, 7(11), 1848-1857.

[2] M. Nemergut, D. Pluskal, J. Horackova, T. Sustrova, J. Tulis, T. Barta, R. Baatallah, G. Gagnot, V. Novakova, M. Majerova, S.M. Marques, M. Toul, J. Damborsky, D. Bednar, Z. Prokop, Y.L. Janin, M. Marek. Illuminating the mechanism and allosteric behavior of NanoLuc luciferase. bioRxiv 2022.

P 16. Characterization of two fumonisin esterases for efficient Fumonisin B1 hydrolysis

Dániel János Incze[1,2], Zsófia Bata[2], László Poppea,[1,3]

inczedani96@edu.bme.hu

[1] Budapest University of Technology and Economics, Department of Organic Chemistry and Technology, Műegyetem rakpart 3, Budapest H-1111, Hungary

[2] Dr. Bata Ltd., Research and Development Laboratory, Bajcsy-Zsilinszky street 139, Ócsa H-2364, Hungary

[3] Biocatalysis and Biotransformation Research Center, Faculty of Chemistry and Chemical Engineering, Babeş-Bolyai University of Cluj-Napoca, Arany János street 11, Cluj-Napoca RO-400028, Romania

Fumonisins are among the most frequently occurring mycotoxins. The most toxic and prominent substance of this mycotoxin family is Fumonisin B_1 (FB_1), which causes 70% of fumonisin contaminations, and different diseases in livestock and humans. [1,2] Fumonisin esterases (FE) catalyze the consecutive de-esterification of FB_1 at the C-6 and C-7 positions, resulting in an aminopentol (hydrolyzed FB_1, HFB_1) and two tricarballylic acid (TCA) molecules as non-toxic final products. [3] We examined two recombinant fumonisin esterases (FE1 and FE2), expressed in *Pichia pastoris*. Contrary to previous hypotheses [3-6], both FEs first selectively hydrolyzed the ester bond at the C-6 position of Fumonisin B1 (FB_1), resulting in the partially hydrolyzed FB_1_7 ($pHFB_1$_7) intermediate, which was transformed into hydrolyzed FB1 (HFB_1). The two FEs had similar and excellent turnover numbers in the first hydrolytic reaction. However, in the second step, FE2 had a 10-fold lower Michelis-Menten constant than FE1, making FE2 the more promising candidate as a decontamination agent of FB_1. Thermal stability of the two FEs investigated with Thermofluor assay revealed a 4.5°C higher melting temperature of FE2 (55.5 °C) than of FE1 (51.0 °C). Homology modeling gave insight into the structural basis that makes these enzymes capable of effectively hydrolyzing FB1 and enabled docking FB_1 into the model structures confirming the regioselectivity of FE1 and FE2. The selectively formed $pHFB_1$_7 could be isomerized into $pHFB_1$_6 via chemical acyl migration; this reaction was accelerated by elevated temperature, polar protic solvents, and alkaline conditions. For the first time, a method for the preparation, isolation, and storage of the isomerically pure $pHFB_1$_7 is reported, which may assist further work toward understanding the toxicity and decontamination of fumonisins.

References:

[1] M. Kamle, D.K. Mahato, S. Devi, K.E. Lee, S.G. Kang, P. Kumar, Fumonisins: Impact on Agriculture, Food, and Human Health and their Management Strategies, Toxins (Basel). 2019, 11, 328.

[2] J. Chen, Z. Wei, Y. Wang, M. Long, W. Wu, K. Kuca, Fumonisin B1: Mechanisms of toxicity and biological detoxification progress in animals, Food Chemistry and Toxicology 2021, 149, 111977.

[3] S. Heinl, D. Hartinger, M. Thamhesl, E. Vekiru, R. Krska, G. Schatzmayr, W.D. Moll, R. Grabherr, Degradation of fumonisin B1 by the consecutive action of two bacterial enzymes, Journal of Biotechnology 2010, 145, 120–129.

[4] S. Masching, K. Naehrer, H.-E. Schwartz-Zimmermann, M. Sărăndan, S. Schaumberger, I. Dohnal, V. Nagl, D. Schatzmayr, Gastrointestinal Degradation of Fumonisin B1 by Carboxylesterase FumD Prevents Fumonisin Induced Alteration of Sphingolipid Metabolism in Turkey and Swine, Toxins (Basel). 2016, 8, 84.

[5] I. Hahn, V. Nagl, H.E. Schwartz-Zimmermann, E. Varga, C. Schwarz, V. Slavik, N. Reisinger, A. Malachová, M. Cirlini, S. Generotti, C. Dall'Asta, R. Krska, W.-D. Moll, F. Berthiller, Effects of orally administered fumonisin B1 (FB1), partially hydrolysed FB1, hydrolysed FB1 and N-(1-deoxy-D-fructos-1-yl) FB1 on the sphingolipid metabolism in rats, Food Chemistry and Toxicology 2015, 76, 11–18.

[6] B. Grenier, H.E. Schwartz-Zimmermann, C. Gruber-Dorninger, I. Dohnal, M. Aleschko, G. Schatzmayr, W.D. Moll, T.J. Applegate, Enzymatic hydrolysis of fumonisins in the gastrointestinal tract of broiler chickens, Poultry Science 2017, 96, 4342–4351.

P 17. Computational Redesign of Industrial Enzymes

Darren Isaacs

d.m.isaacs@rug.nl

Molecular Enzymology, University of Groningen, The Netherlands

My Ph.D. focuses on computationally guided engineering of subtilisin-derived enzymes, specifically those used for the synthesis of peptide-based therapeutics. To engineer these enzymes, computational methods are employed to guide the design of superior variants. We are most interested in enzymes with enhanced characteristics, such as increased ligation activity of peptide fragments. To perform the computationally guided engineering aspect numerous well-known software suites and tools are used, including the Rosetta modeling suite, Yasara, and Foldx. These tools are used in conjunction with in-house developed methods.

One specific subproject has been identifying water tunnels inside the enzyme within the active site vicinity using the Caver PyMol Plugin tool. The intention was to predict the effect stability-enhancing mutations on residues that comprise the water tunnel may exhibit and evaluate any subsequent consequences for enzyme activity. To perform these predictions, we used an in-house protocol known as FRESCO. Several promising mutations were predicted. These mutations were then experimentally tested and screened for activity, and we found that many of the predicted mutations did result in enhanced activity. These included residue positions unreported in the literature.

References:

[1] Toplak, Ana, Eduardo F. Teixeira de Oliveira, Marcel Schmidt, Henriëtte J. Rozeboom, Hein J. Wijma, Linda KM Meekels, Rowin de Visser, Dick B. Janssen, and Timo Nuijens. "From thiol-subtilisin to omniligase: Design and structure of a broadly applicable peptide ligase." Computational and structural biotechnology journal 19 (2021): 1277-1287.

[2] Das, Rhiju, and David Baker. "Macromolecular modeling with rosetta." Annu. Rev. Biochem. 77 (2008): 363-382.

[3] Krieger, Elmar, and Gert Vriend. "YASARA View—molecular graphics for all devices—from smartphones to workstations." Bioinformatics 30, no. 20 (2014): 2981-2982.

[4] Buß, Oliver, Jens Rudat, and Katrin Ochsenreither. "FoldX as protein engineering tool: better than random based approaches?." Computational and structural biotechnology journal 16 (2018): 25-33.

[5] Wijma, Hein J., Robert J. Floor, Peter A. Jekel, David Baker, Siewert J. Marrink, and Dick B. Janssen. "Computationally designed libraries for rapid enzyme stabilization." Protein Engineering, Design & Selection 27, no. 2 (2014): 49-58.

[6] Chovancova, Eva, Antonin Pavelka, Petr Benes, Ondrej Strnad, Jan Brezovsky, Barbora Kozlikova, Artur Gora et al. "CAVER 3.0: a tool for the analysis of transport pathways in dynamic protein structures." (2012): e1002708.

P 18. Photovoltaic enzymes by computational design and directed evolution

Eleftheria Kelefioti Stratidaki[1], Philipp Elbers[1], H. Adrian Bunzel[1,2]

[1] Department of Biosystems Science and Engineering, ETH Zurich, CH-4058 Basel, Switzerland
[2] Centre of Computational Chemistry, School of Chemistry, University of Bristol, Bristol BS8 1TS, UK

Growing global energy demands and pressing needs for sustainable energy solutions call for a reassessment of the ways we generate energy. Solar energy has emerged as a promising candidate. Nevertheless, efficient conversion of solar energy into electrical energy remains limited, as commercial silicon solar cells only reach solar efficiencies of up to 19%. Here, we follow a novel alternative approach to harness solar energy by computationally designing *de novo* heme proteins capable of binding photoactive dye molecules, and improving their photoefficiency through directed evolution.[1] Design is aimed at establishing an electron transport chain within the protein to transfer excited electrons from the dye to heme. Subsequently, the protein facilitates the injection of the electron into the electrode of a photovoltaic cell to increase the generated photocurrents.

In this project, the heme-binding protein is used as a foundation to generate photoenzymes with enhanced activity by: (1) introducing charged tags to locate the protein to the photanode in the solar cell, (2) designing binding sites for different photosensitizer ligands to broaden the spectrum of the absorbed light, (3) including binding sites for electron donor and acceptor ligands to improve the efficiency of the electron transport chain, and (4) applying directed evolution to improve photoefficiency. This strategic approach allows us to tailor the protein's properties and create a system for efficient electron transport, while also gaining insight into the fundamental origins of photocatalysis.

The integration of synthetic biology methods established in this project will enable overcoming current limitations in solar energy conversion, and may be extended to other redox-driven challenges such as H2 production or CO2 fixation.

References:

[1] H.A. Bunzel, J.A. Smith, T.A.A. Oliver, M.R. Jones, A.J. Mulholland, J.L.R. Anderson. Photovoltaic enzymes by design and evolution. bioRxiv 2022.

P 19. Computational approach for enhancing thermostability of pullulanase from *Bacillus* paralicheniformis 9945a

Višnja Kosić, Nataša Božić, Biljana Dojnov, Predrag Banković, Aleksandra Milutinović-Nikolić

University of Belgrade, Institute of Chemistry, Technology and Metallurgy, Njegoševa 12,Belgrade, Serbia

Pullulanase type I (EC 3.2.1.41) from *Bacillus paralicheniformis* 9945a belongs to the GH13_14 subfamily of glycoside hydrolase and is specialized in cleaving (1→6)-α-D-glucosidic linkages in pullulan, amylopectin and glycogen, and in the α- and β-limit dextrins of amylopectin and glycogen. Its application is mainly oriented towards starch modification – in starch saccharification as debranching enzyme, obtaining resistant starches or producing bioethanol. The high cost of pullulanases is a result of their relatively low productivity, and the existing improvements in catalytic efficiency and thermal stability are insufficient for their usage in industrial processes that require high temperatures. Enhancing the thermal stability of pullulanase can be achieved by combining key elements such as increasing surface charge through introduction of specific stabilizing interaction, i.e. additional hydrogen bonds and ionic interactions; increasing the structure hydrophobicity or increasing rigidity of the flexible regions in the protein structure. Various computational strategies that are used today include free energy calculations, disulfide engineering, cavity filling with amino acids with bulkier side chains, altering charge distribution or introducing stabilizing mutations based on computational predictions. It would be of importance to perform simulations to study dynamics of pullulanase at elevated temperatures and identify flexible regions and potential denaturation points. Majority of the modifications aimed at enhancing thermostability of pullulanase were performed on N-terminal domain which is associated with binding of polysaccharide substrates and is important for catalytic activity and stability. Furthermore, suitable combination of multiple strategies might yield the most effective results in enhancing the thermal stability of pullulanase for the potential use in food industry.

Acknowledgements:

This work was supported by the FEBS Youth Travel Fund Grant and Ministry of Science, Technological Development and Innovation of the Republic of Serbia (Grant number: 451-03-47/2023-01/200026).

P 20. Ancestral reconstruction of bacterial pyranose oxidase and C-glycoside oxidase sequence space elucidate unique functional and physiological-relevant properties

Anja Kostelac[1,2], André Taborda[3], Lígia O. Martins[3], Dietmar Haltrich[1]

anja.kostelac@boku.ac.at

[1] Department of Food Science and Technology, BOKU - University of Natural Resources and Life Sciences, Vienna, Austria

[2] Doctoral Programme BioToP - Biomolecular Technology of Proteins, BOKU - University of Natural Resources and Life Sciences, Vienna, Austria

[3] Instituto de Tecnologia Química e Biológica António Xavier, Universidade NOVA de Lisboa, Oeiras, Portugal

The Carbohydrate-Active enZYme (CAZy) database contains catalytic modules and domains of enzymes that degrade, modify, or create glycosidic bonds. In conjunction to the CAZy enzymes, auxiliary activity enzymes provide a helping hand during lignocellulose degradation. The CAZy auxiliary activity family 3 (AA3) belongs to the glucose–methanol–choline (GMC) superfamily of FAD-dependent oxidoreductases. Subfamily AA3_4 is composed of pyranose 2-oxidases. Pyranose oxidase (POx) is an FAD-dependent oxidoreductase catalysing preferably glucose oxidation [1].

C- and *O*-glycosides are naturally found in plants and are known to be metabolized by glycosyltransferases and glycoside hydrolases [2]. In contrast, soil bacteria can metabolize certain *C*-glycosides by a two-step reaction, with the first step being the oxidation of the sugar moiety via oxygen-dependent enzymes. Kumano *et al.* recently reported the characterisation the bacterial enzyme *C*-glycoside 3-oxidase (CGOx), catalysing this first step of *C*-glycoside metabolism by oxidizing the C3 position of the sugar moiety [3].

Taking into account that bacterial POxs show really low affinity to monosaccharides but surprisingly high activity for *C*-glycosides, we decided to elucidate the enzymatic history of ancestral bacterial pyranose oxidases in the light of recent CGOx findings. Both POx and CGOx share the same sequence space [3,4]. We measured kinetic parameters for selected extent enzymes and ancestral enzymes for various monosaccharides as well as glycosides, and performed structural modeling to clarify the mechanism that might have led to the separation of POxs and CGOxs. We hypothesise that the ancestor of pyranose oxidase could thus have mainly functioned for the metabolization of sugar-containing compounds such as *C*- and *O*-glycosides, but later acquired specificity for monosaccharides.

References:

[1] C. Leitner, J. Volc, D. Haltrich. Purification and characterization of pyranose oxidase from the white rot fungus Trametes multicolor. Applied and Environmental Microbiology 2001, 67, 3636-3644.

[2] J. Ati, P. Lafite, R. Daniellou. Enzymatic synthesis of glycosides: from natural O- and N-glycosides to rare C- and S-glycosides. Beilstein Journal of Organic Chemistry 2017, 13, 1857-1865.

[3] T. Kumano, S. Hori, S. Watanabe, M. Kobayashi. FAD-dependent C-glycoside-metabolizing enzymes in microorganisms: Screening, characterization, and crystal structure analysis. Proceedings of the National Academy of Sciences of the United States of America 2021, 118 (40) e2106580118.

[4] A. Kostelac, L. Sutzl, J. Puc, V. Furlanetto, C. Divne, D. Haltrich. Biochemical Characterization of Pyranose Oxidase from Streptomyces canus-Towards a Better Understanding of Pyranose Oxidase Homologues in Bacteria. International Journal of Molecular Sciences 2022, 23 (21), 13595.

P 21. Designing Artificial Fluorinases: Using Unnatural Amino Acids to Desolvate Fluoride Anions

Isabeau Lüddecke, A. G. Jarvis

i.lueddecke@sms.ed.ac.uk

School of Chemistry, College of Science and Engineering, University of Edinburgh, Joseph Black Building, 40 David Brewster Road, EH9 3FJ, Edinburgh, UK

To date, only one enzyme is known in nature that can catalyse the formation of a C-F bond. This is because the fluoride anions that are used as fluorine source are mainly unreactive in an aqueous environment due to their high solvation in water. In 2002, the fluorinase of *Streptomyces cattleya* was discovered.[1] Since then no fluorinating enzymes other than fluorinase homologues have been identified, although it is suspected that there are other fluorinating enzymes.[2] Therefore, it is of great interest to develop new methods for enzymatic fluorination and to create an artificial fluorinase.

Here I present new design strategies to bind fluoride anions in proteins and peptides for nucleophilic fluorination aiming to extend biocatalysis for organofluorine synthesis. The design is based on the active site of fluorinase and urea receptors.[3,4] In view of this, peptides with incorporated unnatural amino acids with urea motifs and specific protein folds were explored to overcome the high desolvation energy of fluoride, with the aim of making them more reactive for the formation of a new C-F bond.

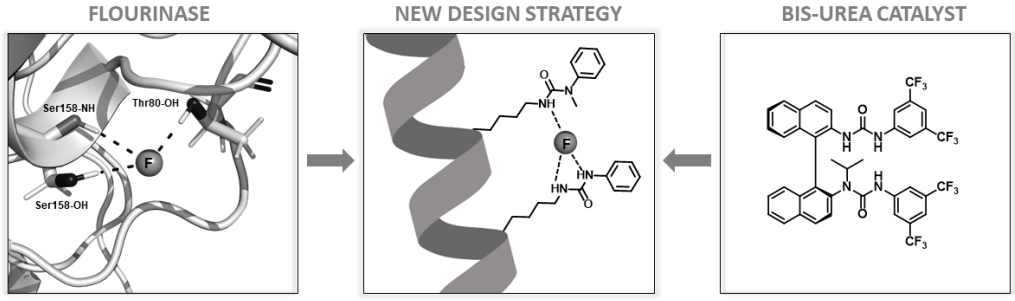


Figure 1. Bioinspired peptide design for fluoride anion binding with unnatural urea amino acids

References:

[1] D. O'Hagan, C. Schaffrath, S. L. Cobb, J. T. G. Hamilton and C. D. Murphy, Biosynthesis of an organofluorine molecule, Nature, 2002, 416 (6878), 279.

[2] Y. Chen, Q. Zhang, X. Feng, M. Wojnowska and D. O'Hagan, Streptomyces aureorectus DSM 41692 and Streptomyces virens DSM 41465 are producers of the antibiotic nucleocidin and 4'-fluoroadenosine is identified as a co-product, Org.Biomol.Chem., 2021, 19 (46),10081–10084.

[3] H. M. Senn, D. O'Hagan and W. Thiel, Insight into Enzymatic C−F Bond Formation from QM and QM/MM Calculations, J. Am. Chem. Soc., 2005, 127 (39), 13643–13655.

[4] 4. F. Ibba, G. Pupo, A. L. Thompson, J. M. Brown, T. D. W. Claridge and V. Gouverneur, Impact of Multiple Hydrogen Bonds with Fluoride on Catalysis: Insight from NMR Spectroscopy, J. Am. Chem. Soc., 2020, 142 (46), 19731–19744.

P 22. Sucrose synthase engineering: towards sustainable glycosylation reactions

Felipe Mejia Otalvaro[1], Gonzalo N. Bidart[1], Alice Branger[2], Ditte H. Welner[1]

felmeo@biosustain.dtu.dk

[1] Technical University of Denmark, Denmark
[2] Ecole Nationale Supérieure de Chimie de Rennes, France

Enzymatic glycosylation represents a feasible biotechnological process to produce industrially relevant compounds with high selectivity and mild conditions.[1] Many glycosylation reactions in nature involve uridine diphosphate (UDP)-dependent glycosyltransferases (UGTs), which utilize expensive sugar donors such as UDP-glucose.[2] Recently, by coupling sucrose synthase (SuSy) with UGTs, a cost-efficient recycling system for UDP-glucose has been achieved.[3] However, enzymatic instability in organic solvents, the low atom economy of the reaction resulting in fructose by-product formation, and the use of sucrose, which competes with food resources, could hinder the successful development of sustainable glycosylation reactions. Therefore, this project aims to design a robust SuSy that efficiently recycles UDP-glucose under green solvent conditions and by using agricultural residues as substrate. To achieve this goal, sustainability assessment and enzyme engineering methods will be implemented.

To improve SuSy thermostability, a semi-rational approach based on consensus sequence mutagenesis and free energy calculations was applied to a plant SuSy. 69 mutations were ranked according to a possible increase in molecular interactions, and the melting temperature and relative activity of a first batch of 28 single mutants were evaluated. 4 out of 28 mutants presented higher melting temperatures without negatively affecting enzyme activity. After combining the 4 mutations, the melting temperature increased by 5 °C, though a decrease of 27% in the relative enzyme activity was also observed. To further enhance the thermostability, a combination of the remaining mutations together with other engineering strategies will be carried out. Additionally, the tolerance to methanol and other green solvents will also be assessed. Nevertheless, these preliminary results highlight the importance of computational enzyme engineering tools to generate small and smart libraries for improving enzyme properties that can facilitate its sustainable application.

References:

[1] B.M. de Roode, M. C. R. Franssen, A. van der Padt, R. M. Boom. Perspectives for the Industrial Enzymatic Production of Glycosides. Biotechnology Progress 2003, 19, 1391-1402.

[2] L.L. Lairson, B. Henrissat, G.J. Davies, S.G. Withers. Glycosyltransferases: Structures, Functions, and Mechanisms. Annual Review of Biochemistry 2008, 77, 521-555.

[3] A. Gutmann, A. Lepak, M. Diricks, T. Desmet, B. Nidetzky. Glycosyltransferase cascades for natural product glycosylation: Use of plant instead of bacterial sucrose synthases improves the UDP-glucose recycling from sucrose and UDP. Biotechnology Journal 2017, 12 (7), 1600557.

P 23. Computer-aided Design of Staphylokinase for Improved Thrombolysis

Jan Mičan[1,2], Veronika Szotkowská[1], Martin Toul[1,2],Veronika Slonková[1], Anton Bushuiev[3], Dávid Lacko[1], Petr Kouba[1,3], Alan Strunga[1,2], Linda Kašiarová[1,2], Dmitri Nikitin[1,2], Jiří Sedlář[3], Josef Šivic[3], Stanislav Mazurenko[1,2], Jiří Damborský[1,2], Martin Marek[1,2], Zbyněk Prokop[1,2], Robert Mikulík[2], David Bednář[1,2]

jan.mican@mail.muni.cz

[1] Loschmidt Laboratories, Department of Experimental Biology and RECETOX, Faculty of Science, Masaryk University, Kamenice 5, 625 00 Brno, Czech Republic,
[2] International Clinical Research Centre of St Anne's University Hospital, Faculty of Medicine, Masaryk University, Pekařská 53, 602 00 Brno, Czech Republic,
[3] Czech Institute of Informatics, Robotics, and Cybernetics, Czech Technical University, Jugoslávských Partyzánů 1580/3, 160 00 Praha 6, Czech Republic

There is a large clinical need to develop more effective, safer, and more accessible thrombolytics to treat ischemic stroke and other thrombotic disorders, which are the leading cause of death and disability worldwide. Staphylokinase is an easy-to-produce bacterial protein that generates fibrinolytic plasmin by facilitating proteolysis of plasminogen, making it an ideal candidate for computer-aided design of an improved thrombolytic. Kinetic studies have shown that increasing the affinity of staphylokinase to plasmin can improve the effectivity of staphylokinase a thousand-fold. In this study, we are using computational design of staphylokinase's protein-protein interface with plasmin to improve its fibrinolytic efficiency. Previously, the first round of design performed using the Rosetta-based AffiLib method yielded the variant SAK01, which showed sevenfold higher plasmin affinity and ten times higher selectivity than wild-type staphylokinase, but achieved only slightly higher fibrinolytic efficiency. In the present study, we have predicted the extended interface of staphylokinase and plasmin using adaptively sampled molecular dynamics, Markov state modeling, and binding energy calculation using the molecular mechanics Poisson-Boltzmann surface area method. Patches of adjacent residues have been targeted for mutagenesis based on scoring by a consensus matrix from a range of predictive methods, including ProteinMPNN, MutCompute, and ESM-1v. The resulting multiple-point mutants have been evaluated by AffiLib binding scores and complemented by an immunogenicity assessment using a machine-learning model trained on a staphylokinase-specific dataset. Additional mutants have been designed by optimization of staphylokinase's sequence to fold into the plasmin-bound conformation using a generative model RFDiffusion. Selected mutants are now being characterized for their fibrinolytic efficiency, binding and selectivity to plasmin, thermostability, and immunogenicity, to identify promising mutants for developing a more effective and safer thrombolytic drug.

P 24. Exploring Light-Emitting Agents in Renilla Luciferases Through Quantum Mechanical Consistent Force Field (QCFF/PI) Method

Ashim Nandi, Aoxuan Zhang, Zhen Tao Chu, Wenjun Xie, Arieh Warshel

Department of Chemistry, University of Southern California, Los Angeles, California 90089-1062, United States

Bioluminescence is a unique phenomenon where living organisms emit light through a specific biochemical reaction. Renilla luciferase (RLuc), sourced from the sea pansy, *Renilla reniformis*, stands out among bioluminescent proteins for its potential as bioluminescent tags, especially given its blue light emission.[1] Recent studies on RLuc8,[2] featuring eight amino acid substitutions, reveal that the emitter coelenteramide can exist in varying protonation states, influenced by proximate proton acceptor residues like ASP in the active site. Through the quantum mechanical consistent force field (QCFF/PI)[3] and the semi-macroscopic protein dipole-Langevin dipole (PDLD-S) method with the linear response approximation,[4] this study confirms that the phenolate and pyrazine states of coelenteramide in WT RLuc8 and its D162A mutant—due to proton transfer to ASP162 and ASP158 respectively—are vital for consistent bioluminescence emission properties. Furthermore, our calculated emission wavelength for the amide ion as emitter in D120A, resulting from chemiluminescence, aligns well with experimental data. The method employed in this study offers cost advantages over QM/MM. Moreover, we introduce a straightforward approach to incorporate the proton transfer reaction coordinate into emission energy calculations, essential for research on proton transfer and light emission.

References:

[1] A. J. Syed & J. C. Anderson. Applications of bioluminescence in biotechnology and beyond. Chem. Soc. Rev. 2021, 50, 5668–5705.

[2] A. Schenkmayerova, M. Toul, D. Pluskal, R. Baatallah, G. Gagnot, G. P. Pinto, V. T. Santana, M. Stuchla, P. Neugebauer, P. Chaiyen, J. Damborsky, D. Bednar, Y. L. Janin, Z. Prokop & M. Marek. Catalytic mechanism for Renilla-type luciferases. Nature Catalysis 2023, 6, 23–38.

[3] A. Warshel. Quantum-Mechanical Consistent Force-Field (Qcff/Pi) Method - Calculations of Energies, Conformations and Vibronic Interactions of Ground and Excited-States of Conjugated Molecules. Israel Journal of Chemistry 1972, 11, 709.

[4] N. Singh, A. Warshel, Absolute Binding Free Energy Calculations: On the Accuracy of Computational Scoring of Protein–Ligand Interactions. Proteins Struct. Funct. Bioinforma. 2010, 78 (7), 1705–1723.

P 25. Development of Homodimeric Papain-like Cysteine peptidases

Ana Obaha, Marko Novinec

ana.obaha@fkkt.uni-lj.si

Faculty of Chemistry and Chemical Technology, University of Ljubljana, Večna pot 113, SI-1000 Ljubljana, Slovenia

Oligomerization is a key strategy that provides proteins with numerous functional and structural advantages over their monomeric counterparts. These far-reaching advantages include increased stability, improved resistance to denaturation and degradation, and refined control over the regulation of enzymatic activity.[1]

Our research focuses on papain-like cysteine peptidases, which are predominantly monomeric enzymes. Our main goal is to develop a method to convert these enzymes into homodimeric configurations by integrating bioinformatics tools and protein engineering techniques. We are primarily focusing on xylellain, which is derived from the bacterium *Xylella Fastidiosa* and was chosen for its ease of production in the bacterial expression system. In addition, we aim to dimerize plant papain-like peptidases, especially ficin and bromelain, which play a central role in biotechnological processes.

Our approach is based on the development of dimeric peptidases based on the optimization of existing protein surfaces to generate interactions that are compatible in geometry and charge. In the initial phase of our study, we focused on identifying plausible interaction surfaces. To this end, we combined insights from crystal structure packing with bioinformatics tools for interaction site prediction, such as SPPIDER[2]. In the case of the model enzyme xylellain, the lower and left sides (regarding the standard orientation) emerged as promising candidates for targeted oligomerization. According to our estimates, these two surfaces are large enough to allow the formation of stable interfaces and are the most hydrophobic.

The surface optimization steps were performed in the laboratory using site-specific saturation libraries followed by screening. The iterative approach has so far led to the identification of two potential homodimer pairs of xylellain, each characterized by hydrophobic residues at their interaction interface. Chimera was used to construct in silico mutants, followed by docking programs to determine potential dimer structures. Prediction of dimeric protein structures was facilitated by protein-protein docking software, including HADDOCK[3] for asymmetric dimer configurations and M-ZDOCK[4] for symmetric counterparts. The stability of these dimers was assessed by molecular dynamics simulations.

In summary, our research highlights the utility of bioinformatics-based protein engineering in modulating enzyme activity and stability through oligomerization. Using a multidisciplinary approach that incorporates bioinformatics prediction and protein engineering techniques, we seek to find new ways to improve the utility of enzymes for various biotechnological applications.

References:

[1] E. D. Levy, S. Teichmann. Structural, evolutionary, and assembly principles of protein oligomerization, Prog. Mol. Biol. Transl. Sci., 2013, 117, 25–51.

[2] A. Porollo, J. Meller. Prediction-based fingerprints of protein-protein interactions, Proteins,2007, 66 (3), 630–645.

[3] G. C. P. van Zundert et al. The HADDOCK2.2 Web Server: User-Friendly Integrative Modeling of Biomolecular Complexes, J. Mol. Biol., 2016, 428 (4), 720–725.

[4] B. Pierce, W. Tong, Z. Weng. M-ZDOCK: a grid-based approach for Cn symmetric multimer docking, Bioinformatics, 2005, 21 (8), 1472–1478.

P 26. Sbv333-TA from *Streptomyces* sp.: discovery, functional characterization and protein engineering

Stefania Patti[1,2], Simone A. De Rose[3], Michail N. Isupov[3], Marta Vanoni[1], Daniela Ubiali[4], Daniela Monti[1], Jennifer A. Littlechild[3], Erica E. Ferrandi[1]

stefania.patti@scitec.cnr.it

[1] SCITEC-CNR, via Mario Bianco 9, 20131 Milan Italy
[2] University of Milan, Via Luigi Mangiagalli, 25, 20133 Milan, Italy
[3] University of Exeter, Exeter, United Kingdom
[4] University of Pavia, Pavia, Italy

Aminotransferases (ATAs, E.C. 2.6.1.x) are pyridoxal-5'-phosphate (PLP) dependent enzymes which catalyze the formation of primary chiral amines by transferring an amino group from an amine donor to a prochiral ketone (acceptor), thus generating a new stereogenic centre at the end of the reaction. ATAs are of great industrial interest for the production of enantiomerically pure chiral amines.

A gene encoding for a putative transaminase was discovered in *Streptomyces* sp. BV333 employing a combined identification technique, which included functional screening and genome mining. The identified gene shared sequence similarities with thermostable ATA sequences, including those from *Thermomicrobium roseii* (43% identity)1 and from hot spring metagenomes (B3-ATA, 41% identity)2. The corresponding enzyme (Sbv333-TA) was successfully produced in *Escherichia coli* co-expressing GroES/GroEL chaperons.

Interestingly, it was found that Sbv333-TA was extremely thermostable, with a melting temperature (Tm) that was only marginally lower (85 °C) than those of the most thermostable transaminases previously reported (87–88 °C).2 Moreover, Sbv333-TA displayed a broad substrate specificity, as far as the amino acceptor spectrum is concerned, and a remarkable activity in the transamination of β-ketoesters, which are rarely accepted by known ATAs.3

Recently, new studies have been carried out to evaluate enzyme stability in the presence of organic (co)solvents. Sbv333-TA proved to be stable in the presence of up to 20% (v/v) of the water-miscible-co-solvents methanol, ethanol, acetonitrile, dimethylsulfoxide, and in biphasic systems with petroleum ether, toluene and ethyl acetate as organic phase. Furthermore, new amine donors were evaluated as possible substrates for this ATA.

This enzyme was also crystallized, and the high-resolution structures of both the native form and the complex with the inhibitor gabaculine were determined. The 3D-structure of the enzyme provided insights on the active site shape and docking studies were performed to evaluate possible modifications to obtain improved variants. Mutagenesis studies are being conducted to broaden the substrate scope of Sbv333-TA

References:

[1] S. Mathew, K. Deepankumar, G. Shin, E. Y. Hong, B. Kim, T. Chung, H. Yun. Identification of novel thermostable ω-transaminase and its application for enzymatic synthesis of chiral amines at high temperature. RSC Adv. 2016, 6, 69257–69260.

[2] E. E. Ferrandi, A. Previdi, I. Bassanini, S. Riva, X. Peng, D. Monti. Novel thermostable amine transferases from hot spring metagenomes. Appl. Microbiol. Biotechnol. 2017, 101, 4963-4979.

[3] E. E. Ferrandi, J. Spasic, L. Djokic, Y. Vainshtein, R. Senthamaraikannan et al. Novel transaminase and laccase from Streptomyces spp. using combined identification approaches. Catalysts 2021, 11, 919.

P 27. Bridging the gap between clinical medicine and chemical physics: multiscale simulation studies of catalytic performance monoamine oxidase A (MAO-A) enzyme and its genetically driven mutants

Martina Rajić, Alja Prah, Janez Mavri, Jernej Stare

National Institute of Chemistry, Ljubljana, Slovenia

Catalytic performance of enzymes is crucial for life processes and deviations from normal performance can lead to diseases or disorders. Among the possible sources of deviations are changes in the enzyme sequence originating from mutations of the part of the genome encoding the enzyme. While a single change in the amino acid sequence may not look like having sizable impact on the performance of the enzyme, this often proves to be otherwise. Namely, a change in an amino acid can alter its interaction with the active site, thereby impacting the catalytic function – particularly if the change involves charged amino acids. In addition, changing an amino acid also affects other interactions within the enzyme, particularly in close vicinity of mutation, thereby affecting its tertiary structure (folding), increasing the possibility of altering catalysis. Herein, we study the genetically driven point mutations E446K and C266F of the monoamine oxidase A (MAO-A) enzyme regulating the levels of serotonin in the central nervous system. These mutations appear to be pathogenic, inducing aggressive behavior and intellectual disability. By investigating the reactive step of serotonin metabolism catalyzed by MAO-A using various techniques of multiscale molecular simulation, among the rest the empirical valence bond (EVB) approach, we find that both mutations lead to sizably increased barriers (from 17.14 kcal/mol in WT to 22.98 and 22.93 kcal/mol in E446K and C266F, respectively). These changes are converted to an approx. 18,000-fold decrease in metabolism rate, which is practically equivalent to a gene knockout (in other words, complete absence of the enzyme). Furthermore, we analyzed the change in electrostatic interactions due to mutation, finding that a substantial part of electrostatic stabilization of the transition state is lost on mutation. This study gives important physical insight into molecular mechanisms leading to neuropsychiatric disorders and the herein employed techniques can potentially be used to predict the tendency towards such disorders directly from the data on genome mutations. Furthermore, the study confirms the hypothesis that the catalytic function of enzymes derives from preorganized electrostatics.

References:

[1] N.J. Bray, M.C. O'Donovan. The genetics of neuropsychiatric disorders. Brain Neurosci Adv 2019, 2.

[2] H. G. Brunner, et al. Abnormal behavior associated with a point mutation in the structural gene for monoamine oxidase A. Science 1993, 262, 578 – 580.

[3] T. M. A., et al. New insights into Brunner syndrome and potential for targeted therapy. Clin Genet 2016, 89, 120 – 127.

[4] A. Prah, D. Pregeljc, J. Stare, J. Mavri. Brunner syndrome caused by point mutation explained by multiscale simulation of enzyme reaction. Scientific Reports 2022, 12, 21889.

P 28. Studying the molecular basics of TLR8 Z-loop proteolytic cleavage by furin protease with an emphasis on the role of water molecules in the system

Maria Bzówka[1,2], Katarzyna Szleper[1], Agnieszka Stańczak[3,4], Tomasz Borowski[5], Artur Góra[1]

Katarzyna.Szleper@polsl.pl

[1] Tunneling Group, Biotechnology Centre, Silesian University of Technology, Gliwice, Poland
[2] Department of Organic Chemistry, Bioorganic Chemistry and Biotechnology, Faculty of Chemistry, Silesian University of Technology, Gliwice, Poland
[3] Institute of Organic Chemistry and Biochemistry, Czech Academy of Sciences, Prague, Czech Republic
[4] Faculty of Science, Charles University, Prague, Czech Republic
[5] Jerzy Haber Institute of Catalysis and Surface Chemistry, Polish Academy of Sciences, Kraków, Poland

Toll-like receptors (TLRs) belong to transmembrane proteins which stimulate a proinflammatory response. TLRs contain three domains: leucine-rich repeats (LRR), transmembrane helix, and cytoplasmic Toll/IL1 receptor (TIR). The general mechanism of TLR signalling involves ligand interaction with the LRR domain, leading to receptor dimer formation or conformational changes in the preexisting dimer. Certain TLRs require the proteolytic cleavage of the Z-loop within their LRRs to perform proper dimer formation and ligand recognition [1]. Furin protease is considered involved in the proteolytic cleavage reaction in TLR8, however, the molecular basis of this process is still unclear.

In our work, we implemented state-of-the-art *in silico* methods including AI-supported protein structure prediction together with molecular dynamics simulations, a small-molecule tracking approach (AQUA-DUCT software [2]), quantum mechanics (Turbomole [3]) and quantum mechanics/molecular mechanics (Gaussian [4]) calculations to investigate the molecular basis for the TLR8 functioning and proteolytic cleavage reaction. Particularly, we focused on the role of water molecules within this complex system. AI-supported protein structure prediction allowed us to obtain an initial TLR8 LRR - furin complex positioned towards each other in a potentially reactive way, where furin's catalytic site residues were close to the proteolytic cleavage site in TLR8. Results from MD simulations combined with those from AQUA-DUCT provided us with a starting point for QM and QM/MM calculations, which enabled the investigation of possible reaction mechanism of furin protease. Thus, we have made the first attempt to describe the reaction profile of Z-loop proteolytic cleavage by furin protease. Also, we showed that QM and MD methods can act as complementary while studying complex biological systems.

Acknowledgements:

The work was supported by the Ministry of Science and Higher Education, Poland from the budget for science for the years 2019-2023, as a research project under the "Diamond Grant" programme [DI2018 014148]. This research was supported in part by PL-Grid Infrastructure (PLG/2021/014751, PLG/2022/015577).

References:

[1] M. Bzówka, W. Bagrowska, A. Góra. Recent Advances in Studying Toll-like Receptors with the Use of Computational Methods. J. Chem. Inf. Model. 2023, 63, 3669–3687.

[2] T. Magdziarz et al. AQUA-DUCT 1.0: structural and functional analysis of macromolecules from an intramolecular voids perspective. Bioinformatics. 2020, 36(8), 2599-2601.

[3] F. Furche et al. Turbomole. WIREs Comput. Mol. Sci. 2014, 4, 91-100.

[4] M. J. Frisch et al. Gaussian, Inc., Wallingford CT, 2019.

P 29. Generating new to nature PHA synthases with conditional variational autoencoder

Tuula Tenkanen, Sandra Castillo, Anna Ylinen, Paula Jouhten, Merja Penttilä

tuula.tenkanen@vtt.fi

VTT Technical Research Centre of Finland Ltd

Many chemicals, polymers, drugs and fuels are still produced with fossil fuel-based chemistry. Producing these substances instead with microbial fermentation would allow more environmentally friendly production, but production levels are often too low or production pathway for the desired product do not exist. Reactions in cells are catalyzed by enzymes. Therefore, enzyme engineering has a key role when aiming to improve microbial production.

Enzymes found in nature are evolved to increase the fitness of the living organisms. Therefore, they might not be optimal for the tasks that we would want them to do. Rational enzyme design can be challenging as the relationships between enzyme structure and function is often not known. Directed evolution allows enzyme development without the need to know these relationships. However, when using directed evolution, the design space is often limited to few mutations to the native sequence used as a starting point [1]. Deep generative models such as variational autoencoders (VAE) can be used to generate new data with similar properties than the training data has. Thus, in recent years the possibility to generate new protein sequences using VAE have been studied by several groups [1, 2, 3]. Hawkins-Hooker *et al.* were able to generate active luciferase enzymes [1]. Furthermore, Sevgen et al. recently reported that they were able to generate new to nature phenylalanine hydroxylases with increased activity [3].

We used a conditional variational autoencoder (cVAE) to produce new to nature PHA synthases. PHA synthase is the enzyme catalyzing polymerization of polyhydroxyalkanoates. Polyhydroxyalkanoates are biodegradable biopolymers that can be used to replace plastic made from fossil fuel.

We trained the cVAE with approximately 10 000 native PHA synthase sequences and 5000 native lipase sequences obtained from Uniprot [4]. The enzyme representation contained contact map calculated with trRosetta [5] and the secondary structure, predicted with PSIPRED [6]. In addition, each amino acid in the sequence was presented with 7 selected physico-chemical features. The model contained self-attention layers and bidirectional recurrent neural networks. As a condition we used the PHA synthase class of each sequence, as PHA synthases can be divided to four classes based on their structure and substrate specificity. After training the model we then generated approximately 10 000 new to nature PHA synthase sequences from class I and selected 16 for wet lab experiments. We then evaluated if the new enzymes were able to polymerize PHA in Saccharomyces cerevisiae. The result showed that two of the new enzymes were active.

References:

[1] A. Hawkins-Hooker, F. Depardieu, S. Baur, G. Couairon, A. Chen, D. Bikard. Generating functional protein variants with variational autoencoders. PLoS Computational Biology 2021, 17 (2): e1008736.

[2] J. G. Greener, L. Moffat, D. T. Jones. Design of metalloproteins and novel protein folds using variational autoencoders, Scientific reports 2018, 8:16189.

[3] E. Sevgen, J. Moller, A. Lange, J. Parker, S. Quigley, J. Mayer, P. Srivastava, S. Gayatri, D. Hosfield, M. Korshunova, M. Livne, M. Gill, R. Ranganathan, A. B. Costa, A. L. Ferguson. ProT-VAE: Protein transformer variational autoencoder for functional protein design. bioRxiv 2023.01.23.525232.

[4] UniProt Consortium. UniProt: the universal protein knowledgebase in 2021. Nucleic Acids Research 2021, 49 (D1), D480-D489.

P 30. Exploring Phenylalanine Ammonia-Lyases: from Protein Engineering to Natural Diversity, for Enhanced Activity towards Challenging Substrates

Tomoiagă Raluca Bianca, Tork Souad Diana, Filip Alina, Levente Csaba Nagy, László Csaba Bencze

raluca.tomoiaga@ubbcluj.ro

Enzymology and Applied Biocatalysis Research Center, Faculty of Chemistry and Chemical Engineering,Babeș Bolyai University Arany János Street 11, 400028 Cluj-Napoca, Romania

Phenylalanine ammonia-lyases (PALs) are highly intriguing biocatalysts that have garnered significant attention due to their remarkable potential in the synthesis of enantiopure phenylalanine analogues, essential building blocks for a wide array of active pharmaceutical ingredients (APIs) and other valuable compounds.[1] The primarily aim of the current study geared towards employing advanced protein engineering and data mining techniques to enhance the activity of phenylalanine ammonia lyase from *Petroselinum crispum* (PcPAL), especially towards challenging substrates, including 3,4-dimethoxy L-phenylalanine — a precursor to L-DOPA, of high pharmaceutical significance.[1]

Rational design strategies were employed to craft variant enzymes that could outperform their wild-type counterparts. The rational design approach proved to be more effective than the saturation mutagenesis method. Both strategies revealed as unique improved variant L134A/I460V, exhibiting modest, yet increased enzymatic activity.

Imprinting the mutational pattern of these variants to PALs of different origins unveiled increased activity in homologs from *Arabidopsis thaliana* (*At*PAL) and *Anabaena variabilis* (*Av*PAL). However, the aromatic ammonia lyase from *Loktanella atrilutea* (*La*AAL), closely related to the recently discovered aromatic ammonia-lyase AL-11 [2], surpassed the engineered variants in the ammonia addition reaction, resulting in superior production of 3,4-dimethoxy L-phenylalanine.[3]

Despite efforts to transfer the distinctive residue panel from *La*AAL to well characterised PALs, there was no observed improvement in activity towards the specified substrate. Conversely, implementing the same rational design methodology from PALs onto LaAAL resulted in decreased activity against the same substrate, implying that distinct customization approaches are required for the novel aromatic ammonia lyases (AALs) as opposed to PALs.

The unique catalytic signature of AALs, namely elevated LaAAL, coupled with its elevated efficacy towards 3,4-dimethoxy L-phenylalanine and high stability, suggest the potential expansion of its substrate panel, encompassing additional aromatic substrates containing electron-donor substituents. These substrates hold significant value but are they are still poorly converted by well-known PALs.

References:

[1] F. Parmeggiani, N.J. Weise, S.T. Ahmed, N.J. Turner. Synthetic and therapeutic applications of ammonia-lyases and aminomutases. Chemical reviews 2018, 118 (1), 73-118.

[2] E.E. Kempa, J.L. Galman, F. Parmeggiani, J.R. Marshall, J. Malassis, C.Q. Fontenelle, J.B. Vendeville, B. Linclau, S.J. Charnock, S.L. Flitsch, N.J. Turner, P.E. Barran. Rapid screening of diverse biotransformations for enzyme evolution. JACS Au 2021, 1 (4), 508-516.

[3] R.B. Tomoiagă, S.D. Tork, A. Filip, L.C. Nagy, L.B. Bencze. Phenylalanine ammonia-lyases: combining protein engineering and natural diversity. Applied Microbiology and Biotechnology 2023, 107 (4), 1243-1256.

P 31. A new-to-nature assimilation pathway for acetyl-CoA

Helena Schulz-Mirbach[1], Philipp Wichmann[1], Ari Satanowski[2], Helen Meusel[2], Tong Wu[2], Maren Nattermann[1], Simon Burgener[1], Nicole Paczia[1], Arren Bar-Even[2], Tobias J. Erb[1]

philipp.wichmann@mpi-marburg.mpg.de

[1] Max Planck Institute for Terrestrial Microbiology, Karl-von-Frisch-Straße 10, 35043, Marburg, Germany
[2] Max Planck Institute of Molecular Plant Physiology, Am Mühlenberg 1, 14476, Potsdam, Germany

Acetyl-CoA is a key intermediate of central metabolism and product of natural and synthetic one-carbon assimilation pathways. Thus, routes that can assimilate acetyl-CoA by conversion into other intermediates of central metabolism are of broad interest. Here, we propose a pathway module for the conversion of acetyl-CoA into pyruvate involving a new-to-nature reaction catalyzed by an adenosylcobalamin (B12)-dependent mutase, which we termed the APRIL pathway (**a**cetyl-CoA to **p**yruvate conversion involving a **L**actyl-CoA mutase). The route proceeds via carboxylation of acetyl-CoA to malonyl-CoA followed by a reduction to 3-hydroxypropioante (3-HP), mimicking parts of naturally occurring carbon fixation cycles. Subsequently, 3HP is converted via 3-HP-CoA to lactyl-CoA with a CoA-transferase and novel L-lactyl-CoA mutase (LCM). Finally, a quinone-dependent dehydrogenase converts L-lactate into pyruvate. The proposed new-to-nature reaction of converting 3-hydroxypropionyl-CoA to L-lactyl-CoA has not been described. We aimed to employ B12-dependent mutases catalyzing structurally related conversions as scaffold enzymes to establish LCM activity. After *in silico* identification of candidate enzymes and determining their activity with lactyl-CoA *in vitro*, we found that a 2-hydroxyisobutyryl-CoA mutase1 from *Bacillus massiliosenegalensis* exhibited the desired promiscuous activity. We assessed the pathway's activity *in vitro* and converted acetyl-CoA to lactyl-CoA via the APRIL pathway. We optimized the pathway *in vitro* by increasing production of 3-HP-CoA using CoA-ligases. Finally, we combined growth-coupled selection in an engineered *Escherichia coli* auxotrophic strain with hypermutation and directed evolution and improved LCM activity 10-fold. With this, we hope to lay the stepping stone for further improvement of an acetyl-CoA utilizing module that could ultimately broaden the engineering perspectives for synthetic metabolism.

References:

[1] Rohwerder, T., Breuer, U., Benndorf, D., Lechner, U. & Müller, R. H. The Alkyl tert -Butyl Ether Intermediate 2-Hydroxyisobutyrate Is Degraded via a Novel Cobalamin-Dependent Mutase Pathway. Appl Environ Microbiol 72, 4128–4135 (2006).

P 32. Rational Design of Thermophilic Chorismate Mutases: Enhancing Enzyme Stability and Functionality for Research and Industry

Ryan Scott Wilkins[1], Bjarte Aarmo Lund[1], Susann Skagseth[1], Adele Williamson[2], Johan Åqvist[3], Bjørn Olav Brandsdal[1]

ryan.wilkins@uit.no

[1] Hylleraas Centre for Quantum Molecular Sciences, University of Tromsø, Tromsø, Norway
[2] School of Science, University of Waikato, Hamilton 3240, New Zealand
[3] Department of Cell and Molecular Biology, Uppsala University, Uppsala, Sweden

Enzymes play pivotal roles in research and industry, but constraints imposed by their natural environments often limit their applications beyond specific conditions. Conventionally, adapting enzymes for laboratory and industrial settings involved random mutations through directed evolution. Leveraging enhanced computational power and deeper insights into enzyme structure-function relationships, we can now employ the rational design of enzymes for optimization suited to specific tasks.

Our study focuses on two main objectives:

1. Characterizing a novel thermophilic chorismate mutase.
2. Rationally designing a thermophilic chorismate mutase using empirical valence bond simulations and structural analysis, guided by the mesophilic *B. pumilus* chorismate mutase and our new template.

Employing a computational framework, we utilize empirical valence bond simulations to scrutinize thermodynamic activation parameters of chorismate mutases, contrasting the mesophilic and thermophilic chorismate variants. We probe the impact of targeted mutations on these parameters, shedding light on essential structural details that bolster enzyme stability and influence these parameters at elevated temperatures. This investigation not only enhances our understanding of enzymes' behavior but also informs the creation of an optimized variant with superior thermophilic properties.

P 33. Computational redesign of substrate selectivity of oxidases with solvent-exposed active sites

Zuzanna Wojdyła, Tomasz Borowski

zuzanna.wojdyla@ikifp.edu.pl

Jerzy Haber Institute of Catalysis and Surface Chemistry, Polish Academy of Sciences, Niezapominajek 8, Krakow, Poland

Oxidases with solvent-exposed active sites typically operate on large polymeric substrates. The location of the active site on a surface of the enzyme not only facilities binding to the surface of the substrate but also provides a stable and highly-mutable scaffold which can serve as basis for further modifications, i.e. such enabling the enzyme to bind small molecules and, in consequence, perform regioselective oxidative reactions.

Herein we present our *in silico* approach to rational redesign of oxidases with solvent-exposed active sites to facilitate binding and selective oxidation of small molecule substrates. The approach involves a combination of constrained MD simulations, followed by analysis of the positions of the substrate that are most probable and favourable for the reaction, with Rosetta enzyme design calculations [1] aimed at stabilisation of the observed position(s) of the substrate. In the next step, for the chosen variants of the enzyme, the feasibility of the substrate binding at the expected pose is assessed with another round of constrained MD simulations and the stability of the variant is tested with an additional simulation for the enzyme without the substrate bound. This approach will provide a library of enzyme variants capable of binding various classes of small molecules.

References:

[1] F. Richter, A.Leaver-Fay, S.D. Khare, S. Bjelic, D.Baker De Novo Enzyme Design Using Rosetta3. PLoS One 2011, 6, e19230.

Author index

Impressum

Name of the course: *FEBS Advanced Course: Computational Approaches to Understanding and Engineering Enzyme Catalysis*
Organizers:
Federation of European Biochemical Societies (FEBS)
Faculty of Science, University of Zagreb, Croatia
Faculty of Biochemistry and Molecular Medicine, University of Oulu, Finland
Croatian Society of Biochemistry and Molecular Biology, Croatia

Place: Department of Chemistry, University of Zagreb Faculty of Science, Horvatovac 102a, HR-10000 Zagreb, Croatia, EU
Date: September 25th–29th, 2023

Organizing Committee:
Aleksandra Maršavelski, Tomica Hrenar, Karlo Sović (Faculty of Science, University of Zagreb, Croatia)
Outi Lampela, Rik Wierenga, Tiila-Riikka Kiema (Faculty of Biochemistry and Molecular Medicine, University of Oulu, Finland)
Jirí Damborský (Loschmidt Laboratories, Masaryk University and the International Clinical Research Center Brno, Czech Republic)
Morana Dulić, Igor Stuparević (Croatian Society of Biochemistry and Molecular Biology)
Course Administrator: Maja Barači
Local organizing staff: Jakov Borovec, Nika Jakobović, Zrinka Pišonić, Marko Preočanin, Filip Sente

Cover page: *Single System Image* supercomputer of Department of Chemistry

Abstracts were prepared by the authors.

Editors: Aleksandra Maršavelski, Karlo Sović, Tomica Hrenar
Publisher: University of Zagreb Faculty of Science
e-ISBN: 978-953-6076-19-2

Sponsors:

Sveučilište u Zagrebu Prirodoslovno-matematički fakultet
e-ISBN: 978-953-6076-19-2