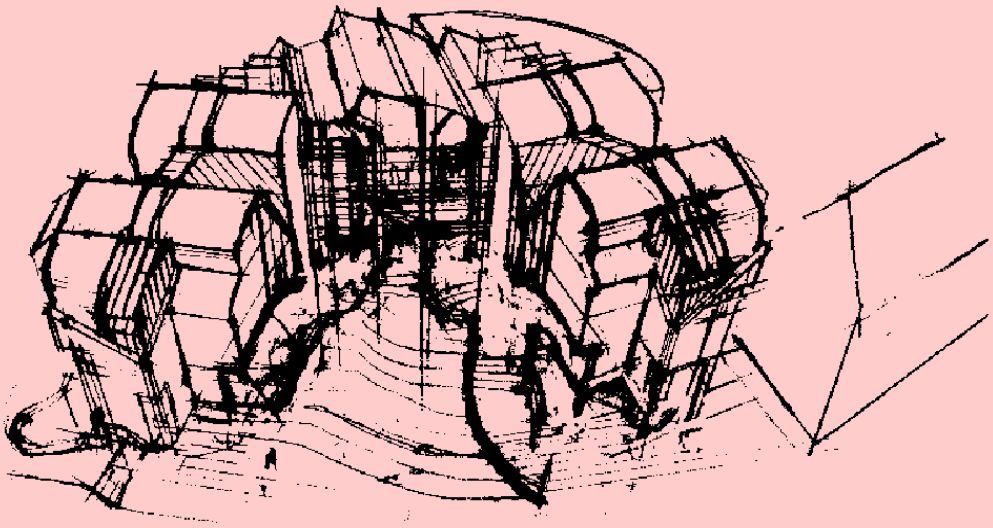
Graz University of Technology
Austria

Institute of Biochemistry



Annual Report 2025

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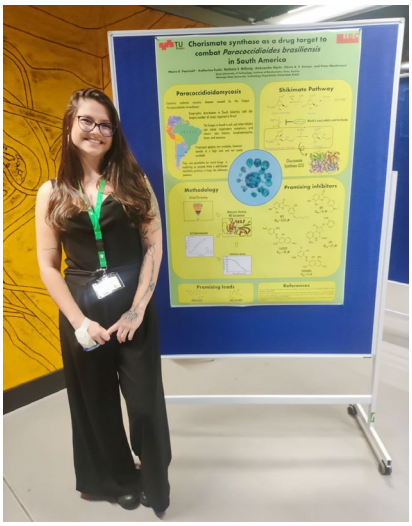
Winkler Group:	Philipp Pelzmann, Christoph Schaffer (both ext. 4528)

Highlights of 2025

The Institute is present on various social media channels! Check out recent updates and highlights by following the QR codes below. The homepage is also constantly updated by Christine Rother. New developments in the groups, job offers, etc. can all be found here: https://www.tugraz.at/institute/ibc/home

Whether you prefer LinkedIn, Facebook or Instagram, we have something for every generation of scientists ...

Macheroux group: In the summer semester, Peter started his research sabbatical at the University of Konstanz to continue a joint project with the Hartig group at the Department of Chemistry. The project revolves around the characterization of NimA, a protein involved in bacterial resistance against nitroimidazol antibiotics. To gain further insight into the mechanism of action of NimA, Peter initiated a collaboration with the electrochemistry group of Prof. Alison Parkin at the University of York, UK. This collaborative effort also led to new research projects at the Graz University of Technology (see project description).



In October, Maira Paetzold from the Universidade Estadual de Maringá (Brazil) joined the Macheroux group. As part of her doctoral research, she is working on a collaborative project in which she tests potential inhibitors of chorismate synthase. In December, Stefanie Baldauf and Maira Paetzold presented posters on their projects at a symposium at the Medical University of Graz. As the photograph shows, Maira has quickly settled both in Graz and within the group and greatly enjoyed participating in the symposium.

Winkler group: In 2025 the WinklerLab welcomed several new members. A former PhD student, Co (in the interest of space), returned after one and a half years as PostDoc in Finland and Lyle (from the Philippines) joined the team as a PhD student. Both started to work on a newly started FWF project (PAT6932824) revolving around phytochrome regulated phosphatases. At the same time two Master students (Dženita and Nedzma) and a Bachelor student (Marie) participated in and eventually completed various projects. When not in the lab, the group enjoyed team building efforts around disc golf and karting events – with the well-deserved relaxed get-togethers afterwards (see picture). Another highlight and substantial team effort, was the "Phytochrome" edition in Methods in Molecular Biology, with three contributions and the editor role within the team.

berdorfer group: 2025 marked a turning point for the Oberdorfer lab. The ERC-Starting Grant ‘HelixMold’ as well as the EIC FET-OPEN project ‘ARTIBLED’ were finished, with lots of students also finishing their respective sub-projects in the corresponding grants. One PhD student and three master students successfully defended their dissertation and master theses, respectively. Several manuscripts were uploaded to preprint servers and will hopefully be fully published soon. Moreover, the labs visibility has become even broader due to our work on de novo designed enzymes and an established protein design pipeline that we call ‘Protflow’. The number of public lectures given by group members, exceeded anything we’ve had in the previous years – a true testament to the impact research from the group has had internationally. The picture shows the ‘Oberdorfer Lab delegation’ at European RosettaCon in September 2025.

echner group: Horst Lechner was awarded a FWF Principal Investigator Project (PAT1730825) from the Austrian Science Fund (FWF), which aims to increase the yield of heterologous proteins from fungal or plant sources in *E. coli* through recombinant methods. Andreas Zechner, a former master's student of Gustav's, was hired as a new PhD student to work on this project.

Christoph Sojer completed part of his master's thesis on this project, attempting to redesign heme-dependent enzymes to enable their production in *E. coli*. Sajith Kolathuparambil is making progress on designing new proteases. Recently entering the experimental phase, he is supported by Master's student Fanni Keserű. He was also selected to present his work by giving a talk at the European RosettaCon 2025 conference in Ljubljana. David Stoll began working on his Master's thesis on the de novo design of Michaelases using the ProtFlow pipeline developed by Adrian and Markus. He also visited Noelia Ferruz's lab in Barcelona to learn more about language models in protein design and molecular dynamics simulations (Erasmus+). Horst had the opportunity to be an instructor alongside Gustav, who was invited to give a lecture at the EMBO Practical Course, "AI for Protein Design," in Puerto Varas, Chile, which was an exceptional experience.

Additionally, we created a group homepage for all news concerning our group:

Biochemistry Group

Group leader: Peter Macheroux
Secretary: Christine Rother
Senior research scientist: Silvia Wallner
Postdoc: Aleksandar Bijelic
PhD student: Stefanie Baldauf
Master students: Thomas Fuchs, Barbara Sophie Millonig
Guest researcher: Maira Gabriela Paetzold (from Brazil)
Project staff: Hannah Gasser
Technical staff: Eva Maria Frießer, Rosemarie Trenker-El-Toukhy

General description

The fundamental questions in the study of enzymes, the bio-catalysts of all living organisms, revolve around their ability to select a substrate (substrate specificity) and subject this substrate to a predetermined chemical reaction (reaction and regio-specificity). In general, only a few amino acid residues in the "active site" of an enzyme are involved in this process and hence provide the key to the processes taking place during enzyme catalysis. Therefore, the focus of our research is to achieve a deeper understanding of the functional role of amino acids in the active site of enzymes with regard to substrate recognition and stereo- and regiospecificity of the chemical transformation. In addition, we are also interested in substrate-triggered conformational changes and how enzymes utilize cofactors (flavin, nicotinamide) to achieve catalysis. Toward these aims, we employ a multidisciplinary approach encompassing kinetic, thermodynamic, spectroscopic, and structural techniques. In addition, we use site-directed mutagenesis to generate enzyme variants to probe their functional role in the mentioned processes. Furthermore, we collaborate with our partners in academia and industry to develop inhibitors for enzymes, which can yield important new insights into enzyme mechanisms and can be useful as potential lead compounds in the design of new drugs.

The methods established in our laboratory comprise kinetic (stopped-flow and rapid quench analysis of enzymatic reactions), thermodynamic (isothermal titration microcalorimetry), and spectroscopic (fluorescence, circular dichroism, and UV-vis absorption) methods. In addition, we use MALDI-TOF and ESI-Q-TOF mass spectrometry, X-ray crystallography, protein purification techniques (chromatography and electrophoresis), and modern molecular biology methods to clone and express genes of interest. A brief description of our current research projects is given below.

Berberine bridge enzyme-like enzymes in plants and fungi

Berberine bridge enzyme (BBE) is a central enzyme in alkaloid metabolism catalyzing the oxidation of the *N*-methyl group of (*S*)-reticuline with concomitant formation of a carbon-carbon bond (the “berberine bridge”) to yield (*S*)-scoulerine. Using bioinformatics, we found that homologs of BBE are widespread among plants, fungi, and bacteria. For example, the model plant Arabidopsis thaliana possesses 27 genes that apparently encode BBE-like enzymes, although the plant does not synthesize complex alkaloids. We have recombinantly produced BBE-like homologs from *A. thaliana* (*At*BBE-like proteins 13 and 15) in *Komagataella phaffii* and identified monolignols and their glycosylated derivatives as potential substrates. We have

solved the X-ray structure of *At*BBE-like 15, and the topology is very similar to that of the BBE from *Eschscholzia californica,* previously solved by Dr. Andreas Winkler and Prof. Karl Gruber. However, the residues that form the active site are distinct from those found in BBE from *E. californica*. The active site architecture of the monolignol oxidoreductase is conserved in approximately half of the BBE-like enzymes, suggesting that this reaction plays an important role in plant metabolism.

To further unveil the role of BBE-like enzymes, we have teamed up with Professor Dr. Tomas Werner from the Institute of Biology at the University of Graz to investigate the *in-planta* functions of the monolignol oxidase sub-family six of the *Arabidopsis* BBE-like enzymes (*At*BBE-like 13, 15, 24, 25, and 26*).* Gene expression analysis employing qRT-PCR has been carried out in all types of plant organs. All genes from subgroup six show expression in roots and flowers. In addition, GUS reporter lines have been generated to investigate gene expression patterns on the tissue level throughout the plant's life cycle. GUS staining patterns have provided valuable information and will serve as a basis for the design of further experiments. Furthermore, we are working on generating single and multiple knockout lines for our genes of interest. Several T-DNA insertion mutants were ordered from the Nottingham Arabidopsis Stock Centre, and homozygous single knockout lines for *At*BBE-like 13 and 15*,* as well as a homozygous double knockout line for the two genes, have been generated. A CRISPR/Cas9 approach is currently underway to generate single, double, and triple knockouts for *At*BBE-like *24, 25,* and *26*. Detailed phenotyping of the single and multiple knockout mutants, combined with the results from the expression analyses (qPCR, GUS plants) and the results of biochemical and physiological studies will provide information for the elucidation of *in-planta* functions of the members of *At*BBE-like subgroup six (thesis project of Reinmar Eggers).

Additionally, we aim to investigate BBE-like enzymes from the model fungus *Neurospora crassa.* During flavoproteome analysis, it became apparent that *N. crassa* possesses not only a high number of core flavoproteins participating in housekeeping reactions but also a substantial number of accessory flavoenzymes required for secondary metabolism [1]. Among these auxiliary flavoproteins, 17 belong to the group of BBE-like enzymes, whereas no information on their exact enzymatic function has been available until now. Therefore, we aim to heterologously express and characterize members of this interesting protein family to deepen our understanding of the function of BBE-like enzymes in fungi. Initial characterization has revealed diverse properties within this enzyme group, including distinct spectroscopic features indicative of different flavin environments, as well as first indications of catalytic activity with simple aromatic substrates for selected candidates. In addition, structural analysis of one enzyme provided insights into active site architecture, supporting the hypothesis of functional diversification within this family. These findings highlight the biochemical diversity of BBE-like enzymes in fungi and provide a foundation for future identification of their native substrates and roles in secondary metabolism (thesis project of Stefanie Baldauf supported by Silvia Wallner).

[1] Kerschbaumer *et al*.: *FlavoFun: Exploration of fungal flavoproteomes* (2022) ***Front Catal***. 2, 1021691.

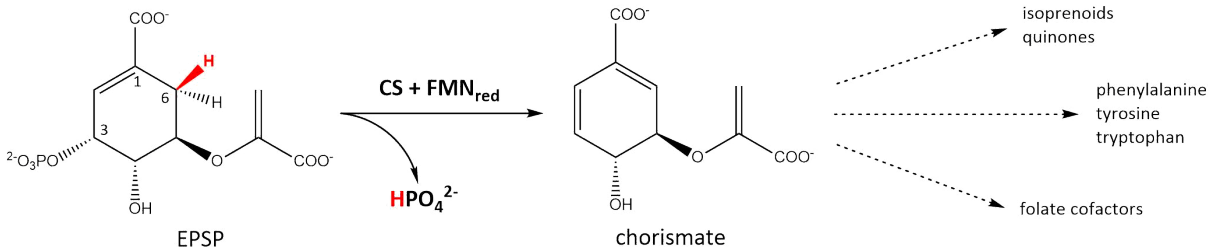
Chorismate synthase as a potential target for pharmaceutically useful inhibitors

Chorismate synthase (CS) is the last enzyme of the shikimate pathway, a biosynthetic route that produces aromatic compounds such as the aromatic amino acids phenylalanine, tryptophan, and tyrosine. As this metabolic pathway is only present in prokaryotes, fungi, and plants, mammals depend on a dietary supply. Inhibition of this pathway has severe consequences for the above organisms because each step of the shikimate pathway comprises an essential reaction in chorismate synthesis, which cannot be bypassed by any alternative enzyme. The absence of the shikimate pathway in mammals makes this metabolic pathway a promising target for developing antibacterial agents and herbicides.

CS was chosen as a drug target because of its unique catalytic mechanism. Potential inhibitors for CS were selected using a combination of virtual screening and molecular dynamics [2, 3]. For the design of new inhibitors, the highly positively charged binding pocket of CS was considered. In the course of testing several compounds, azo-dyes turned out to be the most promising candidates, which was confirmed by various methods. Within the class of azo-dyes 5-amino-3-[(*E*)-(3-chloro-2-hydroxy-5-nitrophenyl)diazenyl]-4-hydroxy-2,7-naphthalenedisulfonic acid showed the best results, which can be explained by the structure of this compound containing several negatively charged substituents that are similarly distributed to those of the natural substrate 5-enolpyruvylshikimate 3-phosphate.

Furthermore, the interaction of the obtained inhibitory compounds with different CSs was evaluated by determining their dissociation constants (1.1-4.4 µM) and IC_{50} values (10-16 µM) with binding and inhibition assays, respectively. To get a better insight into the binding mode, the crystallization of CS-inhibitor complexes is underway.

By investigating the active site of CS, it was observed that the binding pocket contains several invariant arginine residues. However, structural studies of the active site from different CSs showed one arginine residue that differs, suggesting differences in how the substrate is bound to the active site between CSs. To elucidate the binding mode, we need to obtain structural information on more CS-inhibitor complexes. Furthermore, new inhibitory compounds should be synthesized and analyzed to improve their binding to CS (thesis project of Barbara Millonig and Maira Paetzold; supported by Aleksandar Bijelic).



Schematic representation of the seventh and last step of the shikimate pathway. The reaction is catalyzed by chorismate synthase and includes the conversion of EPSP (5-enolpyruvylshikimate-3-phosphate) to the final product of the pathway, chorismate. Chorismate is the starting material for the synthesis of a plethora of aromatic secondary metabolites.

[2] Rodrigues-Vendramini *et al*. *Promising new antifungal treatment targeting chorismate synthase from Paracoccidioides brasiliensis* (2019) ***Antimicrob. Agents Chemother***. 63:e01097-18.

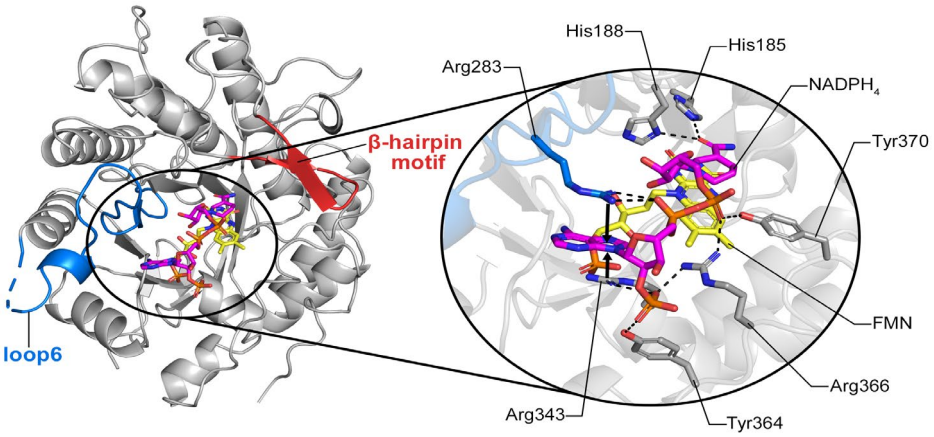
[3] Bueno *et al*. *New inhibitors of chorismate synthase present antifungal activity against Paracoccidioides brasiliensis* (2019) ***Future Microbiol.*** 14:969-980.

Cofactor preference in ene-reductases

Ene-reductases (ERs) are remarkable enzymes since they show high regio-, stereo-, and enantioselectivity and accept a broad variety of alkenes as substrates. The most investigated class of ERs are the oxidoreductases of the old yellow enzyme family (OYEs), which are flavoproteins using a nicotinamide cofactor as hydride donor and a conserved tyrosine residue for proton delivery.

Intriguingly, most ERs prefer NADPH over NADH, constituting a major drawback for industrial applications. In recent years, a broad spectrum of enzymes of the OYE group was investigated in terms of their catalytic cycle and toward their cofactor preference, revealing that all currently known OYEs exhibit the same tertiary structure. However, the origin of the coenzyme specificity remains a mystery.

In order to study the coenzyme specificity, FMN-dependent 12-oxophytodienoate reductase 3 originating from tomato (*Sl*OPR3) has been chosen as a model enzyme. Two different strategies have been chosen to alter nicotinamide cofactor acceptance of the enzyme: (I) rational design in which only single or multiple amino acids have been exchanged and (II) a semi-rational approach, where whole parts of the protein have been swapped with parts from ERs that prefer NADH as cofactor. We recently solved the crystal structures of *Sl*OPR3 in complex with $NADPH_4$ and $NADH_4$ (redox-inactive homologs of NAD(P)H) and revealed that while the binding mode of the nicotinamide group of the cofactors (i.e., above the flavin) is conserved among ERs, their (2'-phosphate)-adenosine tails bind to different sites within the active site cavity [4].



Crystal structure of *Sl*OPR3 in complex with $NADPH_4$: $NADPH_4$ (purple sticks) binds with its nicotinamide group above the flavin (yellow sticks) and with its adenosine tail to a positively charged site within the loop 6 region (blue loop). The key to NADPH preference in *Sl*OPR3 is the electrostatic stabilization of the coenzyme's adenosine tail. The 2'-phosphate group is bound by Arg343, Tyr364, and Arg366 (dashed lines) while the adenine ring is stabilized by cation-π interactions with Arg343 and Arg283 (black arrows).

This structural information is currently being leveraged to design *Sl*OPR3 and morphinone reductase (a NADH-dependent ER) variants with switched cofactor preferences (project of Hannah Gasser). Functional dissection of cofactor preference in ERs revealed that cofactor binding is OYE-class-dependent, with the binding mode observed in *Sl*OPR3 being conserved in plant, fungal, and cyanobacterial lineages [5]. Ancestral reconstruction indicates a stepwise and convergent emergence of the above binding motif. During the course of our studies, we also found that the homodimerization of *Sl*OPR3, previously considered relevant for the enzyme's activity, likely has no physiological significance [6]. Our results raise serious doubts about the physiological role of the so-called self-inhibitory dimer of *Sl*OPR3 in jasmonic acid biosynthesis.

[4] Kerschbaumer, B. *et al.*: *Loop 6 and the β-hairpin flap are structural hotspots that determine cofactor specificity in the FMN-dependent family of ene-reductases* (2024) ***FEBS J.*** 291, 1560-1574. DOI: 10.1111/febs.17055

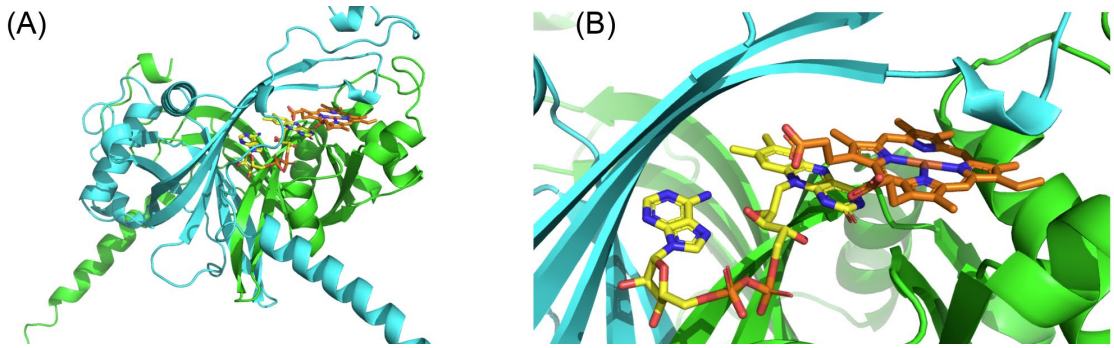
[5] Kerschbaumer, B. *et al.*: *Structural and evolutionary dissection of NADPH-binding motifs in NADPH-preferring ene-reductases* (2026) ***Protein Science***, DOI:10.1002/pro.70521

[6] Kerschbaumer, B. *et al.*: *Analysis of homodimer formation in 12-oxophytodienoic acid reductase 3 in solutio and crystallo challenges the physiological role of the dimer* (2024) ***Sci. Rep.***, 14, 18093. DOI:10.1038/s41598-024-69160-6

NimA – an antibiotic resistance protein with an intriguing cofactor dependence

Nitroimidazole antibiotics, such as metronidazole, dimetridazole, and ornidazole, are widely used to treat bacterial infections in humans and other mammals. Previous studies suggest that resistance to these antibiotics in various bacteria is mediated by *nim* gene products, with NimA (nitroimidazole resistance protein A) binding to different 5-nitroimidazole derivatives.

In collaboration with the group of Prof. Jörg Hartig at the University of Konstanz, Germany, we heterologously produced NimA from *Massilimicrobiota timonensis* in *E. coli* and found that the protein harbors both a FAD and heme cofactor. To date, no information is available on the reaction mechanism of NimA or how these two cofactors cooperate to reduce their respective substrates. Therefore, we initiated a rational mutagenesis approach and generated variant proteins lacking either the flavin or the heme cofactor.



Homology model of NimA: (A) NimA dimer with protomers colored in cyan in green. (B) Close-up view of the cofactors in the predicted NimA dimer. FAD and heme cofactor are shown in yellow and orange, respectively.

These variants are currently being characterized biochemically and electrochemically to elucidate the electron transfer pathways during catalysis. These studies primarily employ UV-vis absorption spectroscopy and pre-steady-state kinetic measurements. In addition, electrochemical analyses are being performed by the group of Prof. Alison Parkin at University of York, UK (master thesis project of Thomas Haas supported by Silvia Wallner and Eva Frießer).

International cooperations

Jörg Hartig, Malte Sinn and Josefine Techel, University of Konstanz, Germany

Alison Parkin and Ella Reid, University of York, UK

Research project

FWF P35858: "The family of monolignol oxidoreductases in *Arabidopsis thaliana*"

Publications

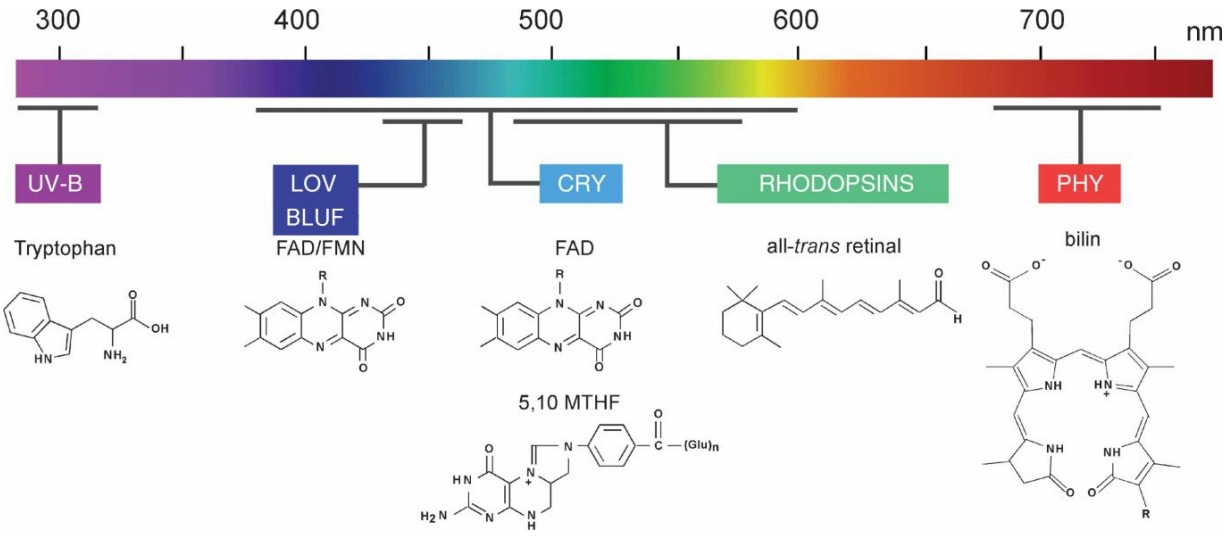
1. Shah, K., Kracher, D., Macheroux, P., Wallner, S., Pick, A., Kourist, R.: *Discovery and characterization of NADH oxidases for selective sustainable synthesis of 5-hydroxymethylfuran carboxylic acid* (2025) ***J. Biotechn.***, 398, 18-28. DOI: 10.1016/j.jbiotec.2024.11.009
2. Krammer, L., Darnhofer, B., Kljajic, M., Liesinger, L., Schittmayer, M., Neshchadin, D., Gescheidt, G., Kollau, A., Mayer, B., Fischer, C. R., Wallner, S., Macheroux, P., Birner-Grünberger, R., Breinbauer, R.: *A general approach for activity-based protein profiling of oxidoreductases with redox-differentiated diarylhalonium warheads (2025)* ***Chem. Sci.***, 16, 6240-6256. DOI: 10.1039/d4sc08454c
3. Pedroni, L., Fuchs, K., Galaverna, G., Macheroux, P., Dellafiora, L.: *Integrated in silico – in vitro study investigating dipeptides as chorismate synthase modulators: spotlight on its mechanism of action* (2025) ***Global Challenges***, 0:e00316, 1-11. DOI: 10.1002/gch2.202500316
4. Abramic, M., Gruber, K., Macheroux, P., Tomic, S.: *Dipeptidyl-peptidase III* (2025) ***Handbook of Proteolytic Enzymes – Metallopeptidases***, Vol. 1, 4th edition, edited by Neil D. Rawlings and David S. Auld, p. 561-570

Photoreceptor Group

Group leader: Andreas Winkler
Postdoc: Cornelia Böhm
PhD students: Oliver Eder, Maximilian Fuchs, Julia Schwekendiek, Lyle Ijssel De Guzman
Master students: Miriam Huber, Nedzma Alijagic, Dženita Kasapović
Bachelor student: Marie Reißenbüchel
Technical staff: Philipp Pelzmann
Apprentice: Christoph Schaffer

General description

The ability to perceive and integrate environmental stimuli is essential for all living organisms. One important ambient factor is light that is sensed by a variety of photoreceptors (Figure below). Many light-responsive proteins are directly connected to enzymatic functionalities and enable photo-activation or -inhibition of various cellular processes. Recently, the interest in light-triggered systems has increased significantly due to the establishment of 'optogenetics', which refers to the concept of genetically targeting biological systems to enable optical control of diverse processes. However, the growing demand for light-controlled systems goes beyond the possibilities of naturally occurring photoreceptors. Even though substantial progress has been made in understanding the concepts of light activation in several photoreceptor families, the rational design of synthetic tools is not straight forward. Since mechanistic descriptions of signal transduction to effector domains differ even within photoreceptor families, it is obvious that a more detailed understanding of the underlying principles of sensor-effector coupling is required.

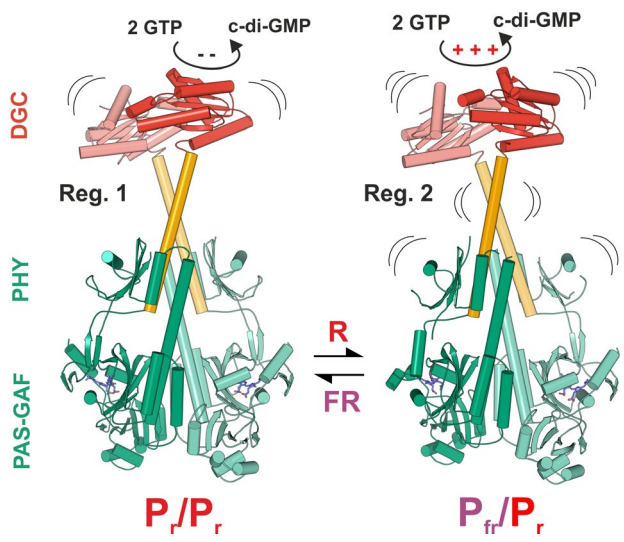


Overview of photoreceptor families and their corresponding cofactors covering the UV/Vis range of the electromagnetic spectrum (adapted from Heintzen WIREs Membrane Transport and Signaling 2012, 1:411–432).

Red-light-regulated diguanylate cyclases

One key approach towards a better understanding of sensor-effector coupling is to appreciate how nature has accomplished its remarkable modularity of sensor-effector combinations. To this end, we focus on light-regulation of cyclic-dimeric-GMP formation by GGDEF diguanylate cyclases that naturally occur covalently linked to various photoreceptors. The understanding of how evolution has accomplished this astonishing modularity of sensor-effector couples is important for the identification of functionally relevant structural elements with the goal of guiding the efficient design of rationally engineered light-regulatable systems.

With the elucidation of the first full-length crystal structure of a naturally occurring phytochrome linked to its functional effector domain, new insights into signal transduction in phytochromes were obtained. The structure revealed a parallel dimeric arrangement of the both domains. Based on an in-depth characterization of the involvement of functionally important elements, our studies provide a foundation for appreciating the fine-tuned coupling mechanism of phytochrome sensors with various effector domains. The characterization of in-solution conformational dynamics substantiates the involvement of several structural elements of phytochromes and highlights their dynamic interplay with the coiled-coil sensor-effector linker region. Our results demonstrate how allosteric light regulation of enzymatic effectors is fine-tuned by the architecture and composition of the coiled-coil linker and by the central helical spine of phytochromes without direct interaction of the sensory module [1].



Additional insights into the mode of phytochrome activation have been obtained by the elucidation of the crystal structure of a constitutively active variant. In particular, the observation of an asymmetric dimer that induces conformational changes in the central sensor-effector linker region allowed us to postulate mechanistic concepts of phytochrome activation that were previously not considered [2]. Understanding the role of structural asymmetry and how it correlates with non-canonical spectroscopic signatures (only partial Pfr formation) in these systems was the focus of the PhD theses of Hieu Tran and Oliver Eder. More recently, we also followed up on this by characterizing a close homolog of *Is*PadC using cryo-EM in collaboration with Elmar Behrmann (University of Cologne).

The role of specific residues in the peculiar light state spectra of *Is*PadC was previously addressed by Hieu Tran (former PhD student). All of these residues being first shell amino acids surrounding the cofactor that are mostly conserved throughout bacteriophytochromes. Since this approach did not allow establishing a prototypical light state spectrum in *Is*PadC, Massimo Totaro and Oliver Eder teamed up to ask the question: “Are there also residues further away from the cofactor that influence optical properties”? To this end, Massimo Totaro used deep learning computational tools (Protein MPNN) to get hints on the functional/structural involvement of residues that appear non-obvious in sequence alignments, but might still have an important role. Oliver Eder together with Stefan Minnich then produced variants probing

identified residues and trying to infer their influence on the spectroscopic properties of the protein variants. Interesting candidates were selected for a detailed characterization involving spectroscopic and HDX-MS measurements. In a nutshell, the identified previously undescribed functional allosteric regulation residues expand our understanding of the intricate regulation mechanisms in the photosensory part of *Is*PadC, but also phytochromes in general. Importantly, the established Function-Structure-Adaptability (FSA) approach, now published in *Structure* [4], can be readily employed for any target protein and it will be interesting to follow the implications it will have in other protein families.

[1] Gourinchas *et al*.: *Long-Range Allosteric Signaling in Red Light-Regulated Diguanylyl Cyclases* (2017) ***Sci. Adv.*** 3, e1602498

[2] Gourinchas *et al*.: *Asymmetric activation mechanism of a homodimeric red light-regulated photoreceptor* (2018) ***eLife***, 7, e34815

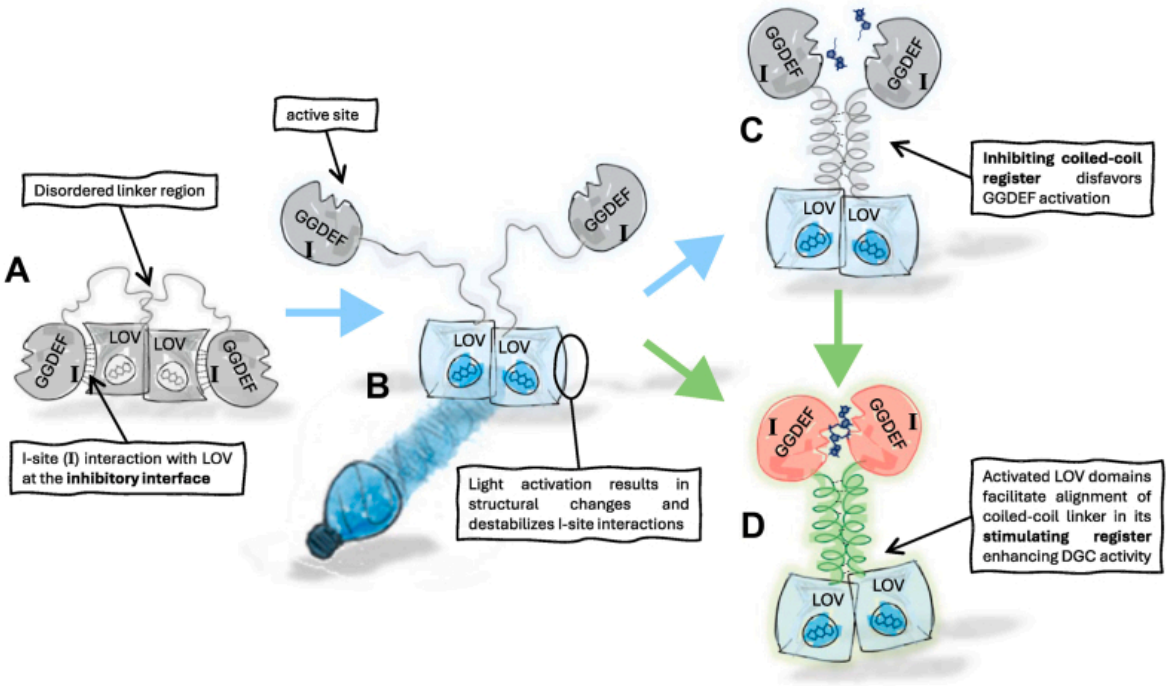
[3] Eder *et al*.: *Integrating Protein Sequence Design and Evolutionary Sequence Conservation to Uncover Spectral Tuning Sites in Red-Light Photoreceptors* (2025) ***Structure***, 33(11), 1916-29

Blue-light-regulated diguanylate cyclases

Photoreceptors sensing blue light were early on identified as key players in plant phototropism, where a specific family of flavin-dependent light-oxygen-voltage (LOV) domains [4] is central to this function. In addition, LOV domains also have potential in applied biosciences, contributing to the field of optogenetics with various LOV-effector couples [5].

In order to complement our efforts in understanding the modularity of sensors regulating diguanylate cyclases described above, we work on LOV-regulated GGDEF domains (PhD projects of Maximilian Fuchs and Julia Schwekendiek). To elucidate molecular mechanisms of light regulation we studied naturally occurring LOV-GGDEF systems and integrated our results with data obtained for other LOV- or GGDEF-containing systems. Since c-di-GMP formation requires appropriate assembly of the active site at the dimer interface of two GGDEF protomers, our focus lied on how such catalytically active dimers are modulated by the sensory domain and the linker region between the two domains. Notably, the linker between LOV and GGDEF domains shows a high conservation of heptad repeats involved in coiled-coil formation. Taken together with the observations for related phytochrome-linked GGDEF systems, the coiled-coil linker is proposed to play an important role in integrating the light signal and activating the GGDEF domains. We employed an integrative structural biology approach combining X-ray crystallography, solution scattering, computational methods and hydrogen-deuterium exchange coupled to mass spectrometry to reveal how a subfamily of LOV-GGDEF proteins, with extraordinary high dynamic range of activation by light, operates on a molecular level [6].

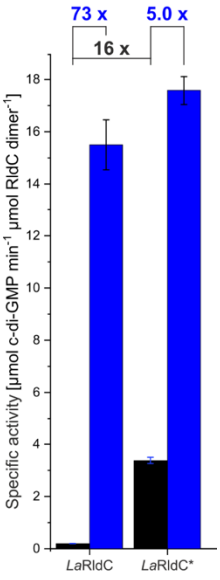
Additional aims of this project went in the direction of addressing the light activated state on a molecular level (finished by Julia Schwekendiek, [7]) and using this system for optogenetics (Maximilian Fuchs and Nedzma Alijagic). Besides focusing on the role of specific residues in the sensor-effector linker and at the inhibitory interface between the two domains, also eliminating feedback product inhibition to improve such optogenetic tools was investigated after testing a range of amino acid substitutions based on a computational tool developed by Massimo G. Totaro [8].



Proposed mechanism of LOV-activated diguanylate cyclase activation. An inhibited dark state conformation (**A**) is activated by illumination (**B**). The inhibitory interface is destabilized and coiled-coil sensor-effector linkers can form in either inhibiting (**C**) or stimulating (**D**) registers. The relative stabilities of these registers influencing the accessible dynamic range and fold-change of the system.

Interestingly, diguanylate cyclases can also be regulated by a variety of other stimuli. Several complex domain architectures like LOV-GGDEF-EAL or Rec-LOV-GGDEF are found in nature. Studying these systems might offer insights into the molecular logic of sensor-effector systems with multiple input or output functionalities (PhD project of Maximilian Fuchs). Our current focus is studying Rec-LOV-GGDEF systems, termed dual-sensor diguanylate cyclases. Next to the light-regulated LOV domain, phosphorylation of the Rec domain provides another layer of complexity. We showed that some of these systems operate as molecular logic gates that affect the regulation of the effector's active or inactive conformations like an OR operator.

An optimized *in vivo* screening that can be used to determine the influence of proteins changing cellular c-di-GMP levels was employed to save the cost and time usually spent in producing and purifying many different homologs. The screening revealed distinct changes in diguanylate cyclase activity between dark and light states, which match changes observed in *in vitro* experiments. One dual-sensor system characterized in detail [9] revealed the characteristics of an OR-gate (see figure on the right; black – dark, blue – illuminated) and is well-behaved to enable further structural and functional analyses. We are currently trying to decipher molecular mechanisms of signal processing in these dual sensor cyclases by structural analyses obtained through X-ray crystallography and HDX-MS. Insights into cooperative domain interplay could eventually facilitate the development of molecular tools for implementing additional regulatory networks in optogenetics.



[4] Losi & Gärtner: Solving Blue Light Riddles: New Lessons from Flavin-binding LOV Photoreceptors (2016) ***Photochem. Photobiol.***, 93, 1, 141-158

[5] Pudasaini *et al.*: *LOV-based optogenetic devices: light-driven modules to impart photoregulated control of cellular signaling* (2015) ***Front. Mol. Biosci.***, 2, 18

[6] Vide *et al.*: *Illuminating the inner workings of a natural protein switch: Blue-light sensing in LOV-activated diguanylate cyclases* (2023) ***Sci. Adv.***, 9, 31, eadh4721

[7] Vide *et al.*: *Coiled-coil register transitions and coupling with the effector's inhibitory site enables high fold changes in blue light-regulated diguanylate cyclases* (2026) ***J. Biol. Chem.***, 302(1), 111020

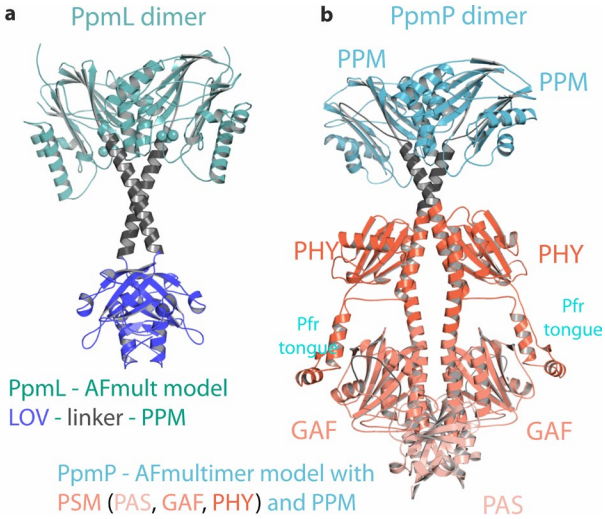
[8] Totaro et al.: *ESM-Scan-a tool to guide amino acid substitutions* (2023) ***Prot. Sci.,*** 33, 12, e5221

[9] Fuchs and Winkler: *Molecular logics in dual sensor regulation of enzyme activity – Phosphorylation OR blue-light activation of cyanobacterial diguanylate cyclases* (2026) ***bioRxiv***, DOI: 10.64898/2026.02.07.704614

Novel Effector Domains to Address Modularity

Recently, the group also ventured into the direction of novel enzymatic output functionalities. To this end, we have initiated efforts to express and purify LOV- (PhD project of Julia Schwekendiek) and phytochrome-regulated PPM phosphatases (Oliver Eder, Miriam Huber, Cornelia Böhm, Lyle Ijssel De Guzman and Dženita Kasapović) to enable a preliminary biochemical characterization of their light regulation capacities. While the expression and purification worked relatively straight forward, the functional characterization is complicated by the fact that PPM phosphatases typically target and dephosphorylate protein substrates with phosphorylated serine or threonine residues. To obtain these substrates, dedicated kinases are needed and current efforts in these projects try to establish routines for the expression and purification of the endogenous protein substrates in the various PPM target proteins.

Preliminary enzymatic characterization of both systems with the phosphatase substrate analog para-nitrophenyl phosphate (pNPP) indicates light regulation capacities in both photoreceptor-PPM families that we term PpmP and PpmL for PPM phosphatase phytochrome and LOV, respectively. Both PpmPs and PpmLs have also proven to dephosphorylate their natural substrate proteins *in vitro*. It will be interesting to follow up on these systems in the future and to compare the role of the sensor-effector linker helices that also appear to form coiled-coils in these contexts. Eventually, this will provide a more detailed understanding of signal integration requirements from the point of the sensor domains, but also identify effector specific requirements.



The figure on the left shows AFmultimer predictions for two representative homologs that highlight the similarities of the LOV-GGDEF and BphP-GGDEF systems described above to the PpmL (panel **a**) and PpmP (panel **b**) proteins. The central coiled-coil (colored in black), which mediates dimerization of the PPM domains and connects sensory modules to the output domains, appears to be key for understanding signal integration in these systems. Interested students are welcome to inquire for available positions in these newly initiated projects.

Mass spectrometry-based services offered

HDX-MS: With the acquisition of a high-resolution time-of-flight mass spectrometer in 2021, the foundations were set to establish the technique of hydrogen-deuterium exchange coupled to mass spectrometry (HDX-MS) in Graz. As one complementary structural analysis technique in the field of integrative structural biology, the idea was to complement existing expertise in Graz and to also offer this methodology to other groups interested in addressing conformational dynamics in proteins. Insights into dynamics and secondary structure stability in different functional states of a protein can provide valuable information as to which regions are involved in a variety of biological processes. The first systems to be characterized included processes like allosteric regulation (see above), biomolecular interactions (with Monika Oberer, UG), immobilization on carriers (with Bernd Nidetzky) and recently also external collaborations with industry partners have started.

Native MS: Another powerful bioanalytical technique that can be performed in our laboratory is native mass spectrometry. Addressing the stoichiometry of protein-protein interactions is frequently an important aspect of a functional characterization of biomolecular complexes. Standard methodologies to address the molecular weight of intact species, such as size-exclusion chromatography, are frequently flawed by the variability of protein structures that often deviate from the spherical approximations used for the mass calibrations. Due to the accuracy of modern mass spectrometry-based techniques, native MS can serve as a powerful alternative to other light scattering-based absolute mass determination techniques, for example, multi-angle light scattering. In addition, also non-covalent interactions with ligands can be identified and provide helpful guidance during the purification of proteins.

Intact mass measurements: An additional standard technique for an initial characterization of newly expressed proteins is the confirmation of its exact mass. Based on this, heterogeneity during sample preparation, amino acid substitutions and/or post-translational modifications can be readily identified. Considering the famous 4th commandment of enzymology „Thou shalt not waste clean thinking on dirty proteins“ (Kornberg, TiBS, 2003), precise knowledge of what is present in your sample preparation can save valuable time in unnecessary follow-up experiments.

Peptide Mass Fingerprints: To further localize specific modifications to specific amino acids in the protein, we employ tryptic digestion and separate the resulting peptides using the HPLC setup connected to our MS. Using the MS/MS capabilities of our systems we can frequently assign the modifications to individual positions and thereby provide additional important insights in the context of protein bioanalytics.

Master Thesis completed

Miriam Huber: *Molecular Mechanisms of Signal Integration in blue light-regulated Diguanylate Cyclases*

Light perception is a fundamental process across all domains of life, enabling organisms to sense and respond to their environment. In this respect, a diverse set of protein families called photoreceptors are key to integrating light signals and trigger specific cellular responses. Hence, they play a crucial role in various biological systems but have also become increasingly relevant in biotechnology, particularly in optogenetics. Among them, bacteriophytochromes are red/far-red light-sensitive photoreceptors that regulate diverse cellular processes through their associated effector domains. However, the functional diversity of these domains remains largely unexplored. Here, we provide first insights into a peculiar subclass of bacteriophytochromes with a metal-dependent protein phosphatase (PPM) as output domain. The latter is generally implicated in dephosphorylation processes essential for bacterial physiology but the exact function of the PPM/ bacteriophytochrome fusion (PpmP) remains unclear. Using AlphaFold2 predictions and phylogenetic data, we identified five promising PpmP candidates and developed an expression and purification strategy based on the Protein Select™ tag (PS-tag). This approach enabled the successful production of three novel PpmPs which, along with previously characterized homologs, formed the basis for further biochemical and biophysical investigations of their distinct absorbance and enzymatic properties. Spectral analysis classified three as non-canonical bathy phytochromes, while two exhibited prototypical characteristics. Thermal recovery kinetics varied among PpmPs, suggesting functional adaptations to distinct physiological requirements. Light-induced changes in oligomerization were observed in one system while others remained constitutively dimeric. Additionally, enzymatic assays confirmed Mn^{2+}-dependent phosphatase activity, with a prototypical PpmP displaying a higher light-dependent dynamic range than a bathy-like system. Crystallization trials for one PpmP yielded promising crystals, though high resolution diffraction data could not be obtained. These findings provide the first photophysical and functional insights into PpmPs, expanding our understanding of their role in bacterial signaling networks. Further research could elucidate their biological relevance and potential applications in optogenetics, advancing our ability to engineer light-controlled signaling pathways.

International cooperations

Elmar Behrmann, University of Cologne, Cologne, Germany

Igor Schapiro, TU Dortmund, Dortmund, Germany

Research projects

FWF PAT6932824: “Signal Integration in Phytochrome-linked PPM-phosphatases”

FWF P34387: “Molecular Mechanisms of LOV-regulated Diguanylate Cyclases”

FWF DOC130: “BioMolStruct – Biomolecular Structures and Interactions” doc.funds (ended October 2025)

Talks at national and international conferences/meetings

1. Fuchs, M.: *Streamlining protein purification using Protein Select$^{(TM)}$*. Cytiva workshop, Vienna, Austria, June 2025
2. Winkler, A.: *Molecular determinants of c-di-GMP formation in sensor-coupled GGDEF domains*. FEMS Micro 2025, Milano, Italy, July 2025
3. Böhm, C.: *Optical control of phosphatase activity in naturally occurring red-light regulated PPM phosphatases*. Phosphatases: from basic research to translation, Würzburg, Germany, July 2025
4. Eder, O.M.: *Cofactor-distant residues tuning phytochrome function.* 16th International Conference on Tetrapyrrole Photoreceptors of Photosynthetic Organisms (ICTPPO), Kaiserslautern, Germany, August 2025
5. Winkler, A.: *Towards Dynamics-driven allosteric regulation in modular sensor-effector systems*. Biophysics Seminar, Graz, Austria, January 2026
6. Winkler, A.: *Bilin-based Photosensory Systems*. GRC on Photosensory Receptors and Signal Transduction, Ventura, U.S.A., February 2026
7. Fuchs, M.: *Double Trouble: Activity Modulation in Dual-Sensor Diguanylate Cyclases*. GRC on Photosensory Receptors and Signal Transduction, Ventura, U.S.A., February 2026

Publications

1. Eder, O.M., Totaro, M.G., Minnich, S.M., Oberdorfer, G., Winkler, A.: *Integrating Protein Sequence Design and Evolutionary Sequence Conservation to Uncover Spectral Tuning Sites in Red-Light Photoreceptors* (2025) ***Structure***, 33(11), 1916-29, DOI: 10.1016/j.str.2025.07.018
2. Winkler, A. *Phytochromes* (2025) ***Methods in Molecular Biology***; Springer Protocols 2970, XII, 356, DOI: 10.1007/978-1-0716-4791-2
3. Fuchs, M. and Winkler, A.: *Hydrogen–Deuterium Exchange Coupled to Mass Spectrometry-Based Analysis of Phytochrome Photoreceptors* (2025) ***Phytochromes: Methods and Protocols***, 2970, 245-65, DOI: 10.1007/978-1-0716-4791-2_15
4. Böhm, C. and Totaro, M.: *Analysis of Sequence Diversity in Subfamilies of Phytochrome-Linked Effectors* (2025) ***Phytochromes: Methods and Protocols***, 2970, DOI: 10.1007/978-1-0716-4791-2_2
5. Eder, O.M., Jančić, M., Winkler, A.: *Strategies for Coexpression of Bilin-Producing Enzymes in Escherichia coli* (2025) ***Phytochromes: Methods and Protocols***, 2970, 29-50, DOI: 10.1007/978-1-0716-4791-2_3
6. Todorović, N., Trifonova, D., Liu, Z., Curin, M., Schooltink, L., Sagmeister, T., Grininger, C., Kiss, R., Gottstein, N., Gesslbauer, B., Winkler, A., Pavkov-Keller, T., Karaulov, A., Valenta, R., Keller, W.: *Major Cat Allergen Fel d 4: Structure and Identification of a Cross-Reactive IgE-Epitope-Containing Area* (2025) ***Allergy***, *early view*, DOI: 10.1111/all.70146

7. Böhm, C., Lehtinen, K., Multamäki, E., Vanhatalo, R., Brander, O., Meier, S.S.M., Rumfeldt, J., Möglich, A., Takala, H.: *Traits of Bathy Phytochromes and Application to Bacterial Optogenetics* (2025) ***ACS Synth. Biol***. 4(8), 3207-3218, DOI: 10.1021/acssynbio.5c00337

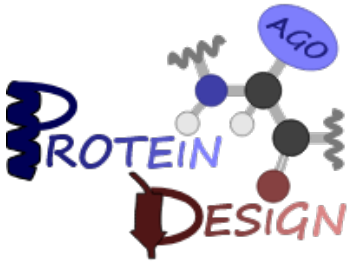
8. Tuure, I., Böhm, C., Rumfeldt, J., Multamäki, E., Takala, H.: *Engineered bacteriophytochrome heterodimers for research and applications* (2025) ***J. Biol. Chem.***, 301(8), 110452, DOI: 10.1016/j.jbc.2025.110452

9. Meier, S.S.M., Hörzing, M., Böhm, C., Düthorn, E.L.R., Takala, H., Uebe, R., Möglich, A.: *Engineering NIR-sighted bacteria* (2025) ***eLife***, 14, RP107069, DOI: 10.7554/eLife.107069

10. Vide, U., Shickle, G., Schwekendiek, J. and Winkler, A.: *Coiled-coil register transitions and coupling with the effector's inhibitory site enables high fold changes in blue light-regulated diguanylate cyclases* (2026) ***J. Biol. Chem.***, 302(1), 111020, DOI: 10.1016/j.jbc.2025.111020

11. Fuchs, M. and Winkler, A.: *Molecular logics in dual sensor regulation of enzyme activity – Phosphorylation OR blue-light activation of cyanobacterial diguanylate cyclases* (2026) ***bioRxiv***, DOI: 10.64898/2026.02.07.704614

Protein Design Group



Group leader: Gustav Oberdorfer
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PhD students: Markus Braun, Wael Elaily, Johannes Peterlechner, Anna Schrüfer, Adrian Tripp, Florian Wieser
Master students: Katharina Buchinger, Melanie Moser, Rebecca Schmid, Tim Seidl, Andreas Zechner
Bachelor students: Martina Tawadrous-Barsoum, Amelia Rieder
Technical Staff: Alma Makic (parental leave), Morakot Chakatok, Claudia Hrastnik, Melanie Moser,
Visiting student: Andrea Borgonovo (MSCA DTN)
Visiting professor: Allon Hochbaum (University of California Irvine) (until July 2025)

General description

De novo protein design is the attempt to use our best understanding of protein biochemistry and biophysics – how proteins fold into their shapes by burial of hydrophobic amino acid residues, or what the typical inter- and intramolecular interactions of amino acids are and how they interact with their environment or targets/substrates – to identify a minimum energy amino acid sequence composition that allows the protein to fold exactly into a desired shape. This is essentially the protein structure prediction problem turned upside down, where a minimum energy structure for a given amino acid sequence is computed. Computationally, protein design represents two interconnected problems: a) How do we score conformations of an amino acid chain and b) How can we sample all its degrees of freedom efficiently? These problems are difficult to solve, because sequence space for a typically sized protein (~200 amino acids) is vast (20^{200}) and comprehensive sampling of it remains a challenge even with current computational power. Besides that, the free energy of such a large system is very difficult to compute with absolute accuracy.

So why try to design a protein, if the odds of success are against the experimenter? With the advent of protein sequence databases and their ever-increasing growth, it has become evident that nature only sampled an infinitesimal small subset of all possible sequences available. Protein design on the contrary allows for the exploration of this 'dark matter' of amino acid sequence space (Figure below). However, the question remaining is: Is it possible to find something new in this pool of unexplored sequences? Given the sheer number of available and yet unexplored sequences, it is reasonable to argue that there are thousands of possibilities for designing novel proteins of high stability and arbitrary shape. All of these bare the potential to go beyond classical biochemical approaches and could ultimately provide solutions to biomedical and biotechnological challenges much faster than nature can. Over the last couple of years, tremendous progress has been made in this direction with many novel protein structures designed from scratch. This can be attributed to advances in understanding the fundamental processes underlying protein folding and concomitant improvements in computational methods. In addition, breakthroughs in the field of synthetic DNA manufacturing and the increase in computational power were key aspects for these successes.

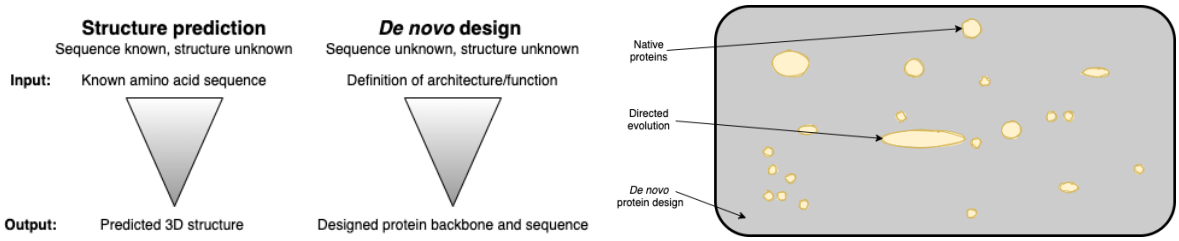

Figure (left) *Differences in structure prediction and de novo protein design* ***(right)*** *Illustrative representation of protein sequence space (grey). Sequence analysis of native proteins shows that tight clusters of protein families can be identified (beige).*

Functionalizing helical *de novo* proteins by deviating from ideal geometries

Coiled-coils, a particular group of protein structures, has seen big advances in terms of design over the last several years. These usually parallel and oligomeric protein assemblies present ideal targets for protein design studies, as they are very regular and follow a repeating sequence, which, in the canonical case, is seven residues long. We could show that it is possible to design genetically encoded, single chain helix bundle structures with atomic level accuracy. To do so, a novel method that uses equations, originally derived by Francis Crick in 1953, which accurately describe the geometries of α-helical protein structures, was established, and used to sample the folding space of helical proteins computationally. The resulting designed proteins were highly idealistic in terms of geometry and showed very high thermodynamic stability (extrapolated $\Delta G_{fold} > 60$ kcal mol^{-1}), with their experimentally determined structures close to identical to the design models and nearly perfect packing of amino acid side chains between the helices. However, it is obvious that in nature, most protein functional sites sit at the end of structural elements or in unstructured regions and therefore are not placed at positions of ideal protein geometry. It has been shown that this can be a result of selective pressure, where the ancestral proteins had more regular structural elements, exhibited higher thermodynamic stabilities and less dynamics, in comparison to their contemporary versions. This is why it is still unclear if idealized protein structures can be functionalized.

In order to address this question, research in the Oberdorfer group is focusing on designing large proteins with topologies not observed in nature. Key elements we hope to find with these studies are whether they exhibit similar rigidity and stability as observed for the small ideal proteins we designed previously. We are also investigating to which extend we can harness, or trade some of the very high thermodynamic stability of these parametrically designed helical bundles to introduce deviations from ideal geometry for the gain of catalytic function. To test different levels of deviation from ideal coiled-coil geometry in helical backbones and to check if this is concomitant with reduction of thermodynamic stability, functional sites of various sizes have been chosen. In particular, the lab is working on metal complexation and cofactor binding (Figure 2). The ability to sample hundreds of thousands of potential protein backbones, which can be used as starting points to introduce catalytic or ligand binding sites into *de novo* designed helical proteins is a big advantage over previous attempts in designing functional proteins. Initial results from this research shows that there might be a tradeoff between high stability and degree of idealism as far as the protein backbone is concerned, however, many more designs have to be made and characterized before we can draw definite conclusions. In answering these questions though, we hope to pave the way for downstream applications of *de novo* protein design to biocatalysis, biomedical and biotechnological problems.

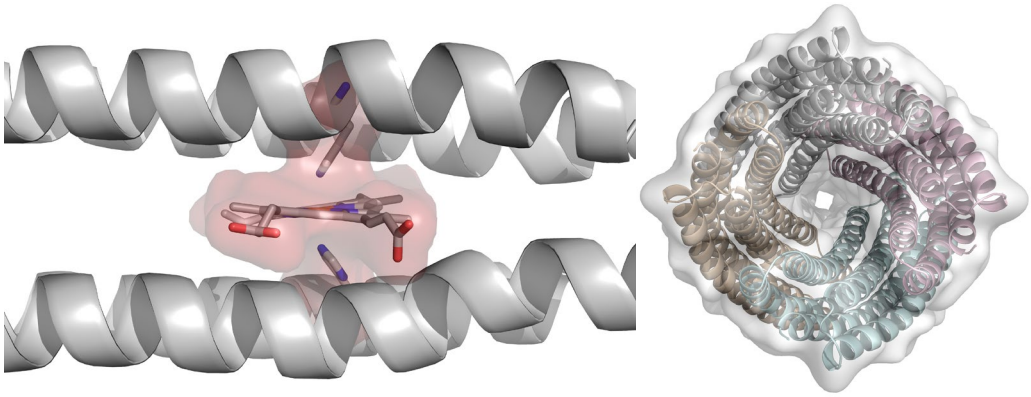

Figure 2. (left) *Computationally designed 4-helix bundle with a designed binding site for b-type heme. It can clearly be seen how much the otherwise straight helices had to be bent, to accommodate the heme cofactor.* ***(right)*** *top view of a computationally designed 20-helix bundle. This is a completely unknown topology to nature.*

Master Theses completed

Melanie Moser: *Redesign of a de novo homo hexameric protein pore for crystallisation*

The master's thesis, explores the intersection of computational protein design and wet-lab structural analysis. The overarching objective of the research is to engineer and modify specific proteins to achieve stable structures that can be successfully analyzed using X-ray crystallography. The thesis is divided into three distinct research projects:

Project 1: Surface Redesign of a Homo Oligomeric Nanopore The first project focused on "3H22C6," a de novo hexameric protein pore initially designed by Gustav Oberdorfer, which has potential applications in nanopore DNA sequencing. To obtain a crystal structure without altering the functional hydrophobic core of the pore, the author attempted a targeted surface redesign. The computational workflow utilized Rosetta ResidueSelector to isolate surface residues, ProteinMPNN for sequence generation, and AlphaFold 2 for 3D structure prediction. Six selected sequences were cloned into an E. coli vector featuring an N-terminal His-Tag and a TEV-cleavage site. Despite extensive wet-lab efforts—including protein expression, purification, Small-angle X-ray scattering (SAXS), and mass spectrometry—the redesigned proteins proved highly unstable, particularly after the His-Tag was cleaved. The purification process was plagued by protein precipitation and the appearance of unexpected, unidentified bands on SDS-PAGE gels. Although one crystal was successfully grown, it shattered during handling and could not be analyzed at the synchrotron, leaving the structural analysis incomplete.

Project 2: Full Redesign of the 3H22C6 Because the surface redesign failed to yield a stable, crystallizable protein, the second project aimed for a complete redesign of the 3H22C6 pore. The computational approach was modified to use Rosetta Relax, ProteinMPNN, and AlphaFold Multimer over three iterative design cycles. A Rosetta BUNS filter was also applied to eliminate unsatisfied hydrogen bonds. Two top sequences were selected for wet-lab testing. Unfortunately, similar wet-lab challenges occurred; the fully redesigned proteins precipitated completely during the TEV cleavage step required to remove the His-Tag. Ultimately, these protein variants were too unstable to even reach the crystallization phase.

Project 3: Crystallisation of the BocKRS (A) The final project shifted focus away from the de novo pore to a variant of Pyrrolysyl-tRNA Synthetase (PylRS), an enzyme capable of incorporating non-canonical amino acids. Initial attempts to express and purify the full-length protein failed because the protein was highly unstable with both its N- and C-terminus intact. To resolve this, the protein was successfully recloned to remove its N-terminal region. This modification stabilized the protein, allowing for successful expression in an E. coli Rosetta 2 strain and subsequent purification. After screening multiple conditions, a successful crystal was grown using a specific Molecular Dimension BCS condition. The crystal structure was solved at a resolution of 2.7 Å and uploaded to the Protein Data Bank under the ID 8RIS. The solved structure revealed the protein in an APO (open loop) state, contrasting with the closed loop of its template structure (2ZIM).

Overall, the thesis demonstrates the complexities of protein engineering and structural biology. While the de novo 3H22C6 pore struggled with severe instability upon His-Tag removal, the strategic truncation of the BocKRS (A) synthetase overcame initial instability, leading to a highly successful structural characterization and a new PDB entry.

Andreas Zechner: *Solubilizing Membrane Proteins through AI based Protein Design*

The master's thesis focuses on overcoming the severe limitations in studying and applying membrane proteins—specifically their poor solubility, high hydrophobicity, and reliance on stabilizing detergents. The primary objective was to computationally redesign two membrane enzymes, Alkane Monooxygenase B (AlkB) and Human Duodenal Cytochrome B (DcytB), to create water-soluble variants that maintain their proper folding and catalytic functions. Redesign of Alkane Monooxygenase B (AlkB) AlkB is a bacterial diiron enzyme involved in alkane oxidation. The redesign aimed to enhance its solubility without disrupting its native active site or its interaction interface with the electron donor protein AlkG. Utilizing AI tools like ProteinMPNN, AlphaFold 3, ESMFold, and Rosetta FastRelax, the author successfully engineered several variants with increased hydrophilic surface residues while preserving the overall protein fold. Experimental validation via Circular Dichroism (CD) spectrometry demonstrated that the designed proteins exhibited stable folding, general structural integrity, and reversible thermal denaturation, maintaining stability up to 75 °C in the presence of iron. While UV-Vis and CD results suggested possible iron binding, structural analyses indicated that the proper orientation of active-site histidine residues remains a challenge, requiring further fine-tuning to fully secure enzymatic activity.

Redesign of Human Duodenal Cytochrome B (DcytB) DcytB, a human duodenal iron reductase essential for dietary iron uptake, was targeted to overcome solubility constraints. The redesigned variants, termed "Irozymes", initially faced structural instability in a specific loop, which was successfully resolved using RFdiffusion Fold Conditioning. Experimental evaluation identified two highly promising variants, I4 and I5. These designs achieved proper folding and successfully incorporated necessary heme cofactors, which was confirmed by mass spectrometry and UV-Vis measurements. Most notably, qualitative activity assays confirmed that I4 and I5 exhibited functional ferric reductase activity. Variant I4 demonstrated the highest initial water solubility, though it experienced some precipitation over time, indicating a need for further stability optimization.

De Novo WD40 Metalloenzymes (Side Project) A complementary side project explored the design of artificial metalloenzymes using a highly stable de novo WD40 protein scaffold, a structure that naturally acts only as a protein-protein interaction scaffold and lacks inherent catalytic activity. Using LigandMPNN, the project successfully generated highly stable coordination interfaces for both Cerium (Ce(III)) and diiron centers within the scaffold's central channel. While computational models for specific substrate binding pockets showed promise, establishing a stable protein-protein interface for the coenzyme AlkG proved computationally challenging and requires further development.

In conclusion, the thesis serves as a successful proof-of-concept, demonstrating the tremendous potential of AI-guided protein engineering in tackling major challenges in synthetic biology. It proves that the functional solubilization of complex membrane-associated enzymes is achievable and establishes a robust computational framework for the future development of both redesigned membrane proteins and novel artificial metalloenzymes.

Doctoral Thesis completed

Wael Elaily: *Computational Design of Single Chain Helical Barrels and Their Functionalization*

Designing a detailed, customized de novo protein with a unique topology that is both water-soluble and functionalized as a biocatalyst is a multifaceted and challenging task. Previously, de novo helical bundles have emerged as promising candidates, offering exceptional design accuracy and stability enabled by parametric design using Rosetta. Here, we explore for the first time the feasibility of designing a single-chain six helical bundle that forms a totally straight barrel. This novel fold creates an open hydrophobic channel capable of accommodating and binding substrates, paving the way for efficient biocatalysis.

The initial design, 6H5L, was successfully produced in E. coli, exhibiting remarkable structural integrity and thermodynamic stability even up to 95°C and more than 20% DMSO. Different characterization methods confirmed precise folding, with the crystal structure showing an exceptional match to the design model. This validated the barrel fold architecture, which creates an open hydrophobic channel stabilized by two well-defined rings of polar residues, each containing three Lys and three complementary Glu forming salt bridges. Notably, 6H5L showed retro-aldolase activity, confirming the nucleophilic activity of lysine residues in the hydrophobic channel.

Through rational and computational optimization, the active site was redesigned, resulting in several enhanced variants, with 6H5L_199K achieving a 10-fold increase in activity. 6H5L and 6H5L_199K demonstrated a second dehydration step following the aldol reaction with naphthaldehyde derivatives or cinnamaldehyde substrates, producing α,β-unsaturated ketones. Both designs effectively catalyzed other reactions, including the Michael addition. Remarkably, 6H5L also exhibited alanine transaminase activity in its holo-protein form bound to PLP, making it the first de novo biocatalyst with this functionality.

Using RosettaRemodel, we successfully designed a truncated version, 6H5L_Cut, which exhibited a crystal structure with high matching over the predicted AF2 model. Further improvements were achieved using ProteinMPNN, which enhanced the protein’s producibility by 10-fold, resulting in the new variant 6H5L_mpnn1740. Both new versions demonstrated

stable folding and retained catalytic activity. Exceptionally, our de novo six-helical barrels stand as robust and versatile proteins with exceptional stability, mutant resistance, and adaptability for reengineering. Their full-length version features two separate catalytically active cavities at both ends, supporting promiscuous biocatalysis and cascade reaction optimization. Additionally, the truncated version connects the two cavities, enabling substrates interaction at the core and allowing more complex reactions, including coenzyme-dependent ones. Altogether, these new de novo barrels serve as ideal protein reaction chambers, both in pure form and whole cell biotransformations, with transformative potential for industrial biocatalytic applications.

International cooperations

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Research projects

ERC StG (GA: 802217): "HelixMold: Computational design of novel functions in helical proteins by deviating from ideal geometries" (ended April 2025)

ERC FET-OPEN (863170): "Artificial fluorescent proteins for the next generation of sustainable and safer LEDs" (ended January 2025)

FWF DOC130: "BioMolStruct – Biomolecular Structures and Interactions" doc.funds (ended December 2025)

TUG – LEAD "DigiBiotech - Learn and Predict: Digitalization of Biotechnology"

Talks at national and international conferences/meetings

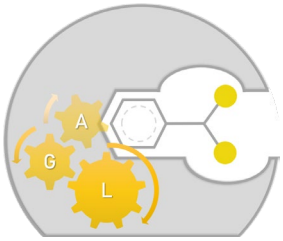
1. Oberdorfer, G.: *De novo Enzyme Design by Artificial Motif Library Scaffolding,* ETH Zürich, Zürich, Switzerland, March 2025
2. Oberdorfer, G.: *Modelling and expression of de novo coiled-coil peptides,* Marseille, France, March 2025
3. Oberdorfer, G.: *A hybrid machine learning and atomistic modeling approach for the design of de novo enzymes,* SynMicroMeeting, Marburg, Germany, April 2025
4. Oberdorfer, G.: *A hybrid machine learning and atomistic modeling approach for the design of de novo enzymes.* The Protein Society Meeting, San Francisco, USA, June 2025
5. Oberdorfer, G.: *A hybrid machine learning and atomistic modeling approach for the design of de novo enzymes.* Benzon Meeting, Copenhagen, Denmark, September 2025
6. Oberdorfer, G.: *A hybrid machine learning and atomistic modeling approach for the design of de novo enzymes.*, European RosettaCon, Ljubljana, Slovenia, October 2025

7. Oberdorfer, G.: *A hybrid machine learning and atomistic modeling approach for the design of de novo enzymes.* EMBO AI 4 Protein Design Workshop, Puerto Varas, Chile, November 2025
8. Oberdorfer, G.: *A hybrid machine learning and atomistic modeling approach for the design of de novo enzymes.* Freitagsseminar Institute of Chemistry, Graz, Austria, December 2025

Publications

1. Braun, M., Tripp, A., Chakatok, M., Kaltenbrunner, S., Totaro, M., Stoll, D., Bijelic, A., Elaily, W., Hoch, S. Y., Aleotti, M., Hall, M., Oberdorfer, G. *Computational enzyme design by catalytic motif scaffolding* (2026) ***Nature*** 649, 237–245. DOI: 10.1038/s41586-025-09747-9
2. Wieser, F., Kaltenbrunner, S., Zechner, A., Zach, M., Pock, T., Oberdorfer, G. *ELEN – Predicting Loop Quality in Protein Structure Models* (2025) ***bioRxiv*** 2025.08.07.668890. DOI: 10.1101/2025.08.07.668890
3. Vornholt, T., Stockinger, P., Mutný, M., Jeschek, M., Nestl, B., Oberdorfer, G., Osuna, S., Pleiss, J., Welner, D., Krause, A., Buller, R., Ward, T. *Of revolutions and roadblocks – the emerging role of machine learning in biocatalysis* (2025) ***ACS Cen Sci***. DOI: 10.1021/acscentsci.5c00949
4. Eder, O., Totaro, M., Minnich, S., Oberdorfer, G., Winkler, A. *Integrating protein sequence design and evolutionary sequence conservation to uncover spectral tuning sites in red-light photoreceptors* (2025) ***Structure*** 33, 1–14. DOI: 10.1016/j.str.2025.07.018
5. Nigl, A., Delsoglio, V., Sovic, L., Grgić, M., Malihan-Yap, L., Myrtollari, K., Spasic, J., Winkler, M., Oberdorfer, G., Taden, A., Anić, I., Kourist, R. *Engineering of Transmembrane Alkane Monooxygenases to Improve a Key Reaction Step in the Synthesis of Polymer Precursor Tulipalin A* (2025) ***Angew. Chem. Int. Ed.*** 64, 25, e202503464. DOI: 10.1002/anie.202503464
6. Elaily, W., Stoll, D., Chakatok, M., Aleotti, M., Grill, B., Lechner, H., Hall, M., Oberdorfer, G. *Computational design of a thermostable de novo biocatalyst for whole cell biotransformations* (2024) ***bioRxiv*** 2024.10.07.617055. DOI: 10.1101/2024.10.07.617055

BioTechMed YoungResearcherGroup “Enzyme Design”



Group leader: Horst Lechner
PhD students: Sajith Kolathuparambil, Andreas Zechner
Master students: David Stoll, Christoph Sojer, Fanni Keserű
Technical Staff: Claudia Hrastnik

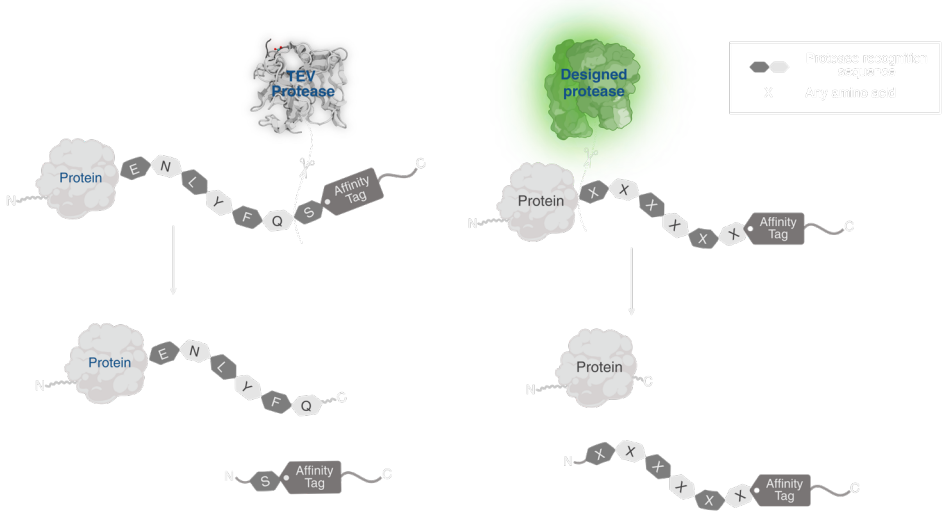
General description

Enzyme engineering is often characterized by its tedious, time-consuming and labor-intensive nature. Computational enzyme engineering and *de-novo* design can be supportive to increase the success rates and speed up the process. One of the reasons for recently grown interest to apply more computational methods in this area is a consequence of the recognition that AI methods can be applied to many challenges in this field and new, powerful methods were developed over the last few years. We use those programs to design new enzymes or redesign existing ones to change their properties.

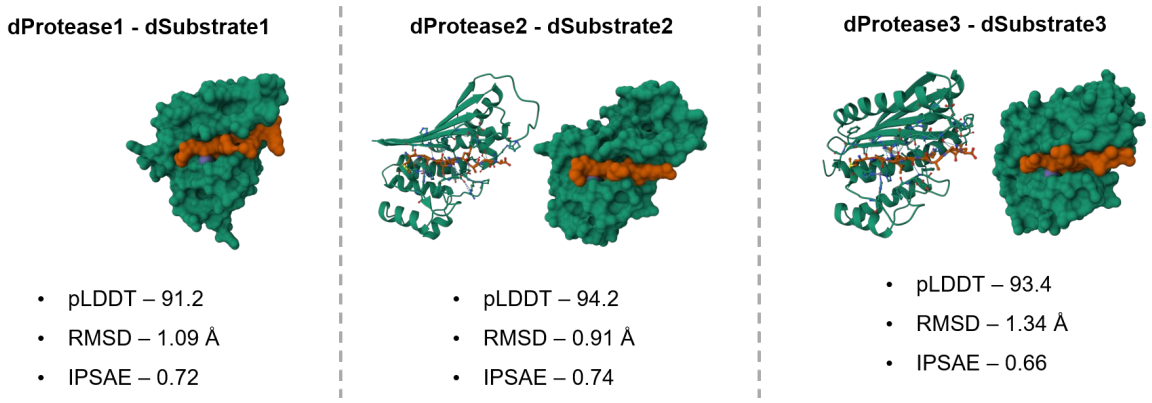
Design of new proteases

Efficient recombinant protein purification is pivotal in biological research as well as in biotechnology, often relying on affinity tags. However, complete removal of C-terminal tags is problematic since most endoproteases cut towards the C-terminal end of their recognition sequence. Nonetheless, the additional residues on the recombinant protein from the protease recognition sequence after protein tag removal can be detrimental to the proper folding, activity or crystallization of the target protein.

We want to develop a sequence-specific proteases tailored for C-terminal tag removal without leaving any residues from the recognition sequence, a challenge unmet by currently used proteases. Therefore, we harness the power of *de novo* computational design to create new protein backbones using RFDiffusion (Watson et al., 2023), harboring a catalytically active fragment of a known protease and a binding cleft for the target sequence. ProteinMPNN (Dauparas et al., 2022) is used to derive sequences for these backbones. We ordered almost 30 different genes and most of them appeared to be active in a newly developed assay based on a split-GFP. These designs are currently analyzed more deeply using FRET-FP pairs.



Enzyme design approach. Left: Currently available proteases (as example the Tobacco Etch Virus (TEV)-protease) cleave after their recognition site leaving their recognition sequence on the target protein. Right: Proposed protease cleaving before its recognition sequence.



Designed proteases (green) with their peptide substrates (orange)

Yield enhancement of recombinant enzyme production

Many natural proteins that have biotechnological or pharmaceutical applications cannot be produced in the desired quantities, or are only stable to a limited extent. This results in higher production costs and a larger ecological footprint. This project is testing computer-aided methods to increase the stability and yield of production. These methods have been trained using machine learning to predict which amino acids the building blocks of proteins need to be replaced to increase stability and yield while maintaining functionality and structure. Using two model proteins, we will evaluate different methods for designing and evaluating the designed sequences, and identify the most effective methods. A set of designed proteins, computed and selected using various methods, will be produced in bacteria and tested for properties such as stability and activity. Building on this knowledge, our aim is to develop a general method for modifying amino acid sequences to increase protein yields.

Ahmed Alagic started as student employee to evaluate computational protein design strategies to improve expression yields of hard-to express proteins. He focused on unspecific peroxygenases and this work was continued by Christoph Sojer who worked with unspecific peroxygenases as well as an P450-enzyme and is now in the hands of Andreas Zechner.

Design of Michaelases

David Stoll pursued de novo design of enzymes for an intermolecular Michael addition by adapting the Riff-Diff workflow for his Master thesis. Starting from a theozyme inspired by a recently evolved Michaelase (derived from deoxyribose-phosphate aldolase variants), we encoded key catalytic features, electrophile activation by a lysine and stabilization of reactive intermediates and sought protein scaffolds able to enforce them.To build and screen candidates at scale, we combined diffusion-based scaffold generation with a neural network for sequence design, followed by geometric and energy filters. We introduced an evaluation step using a GNINA-derived docking metric to enrich for binding modes consistent with productive substrate placement, and used molecular dynamics to rank designs by their ability to maintain near-attack geometries. A curated set of Michaelase designs was expressed in *E. coli* and purified by Ni-affinity and size-exclusion chromatography. All were soluble, predominantly monomeric, and several showed the intended all-α helical content by circular dichroism, indicating that the global fold objective was frequently met. However, a turn-on probe assay and HPLC failed to detect activity, whereas a DERA-MA positive control responded robustly. To validate structural accuracy, we solved the crystal structure of one design (MIC8). The overall fold matched the model, but residues 70–95 in the active site lacked electron density, suggesting local disorder. These results show Riff-Diff reliably delivers stable folds, but effective Michael catalysis will require tighter active-site preorganization, strategic electric fields, and ensemble-based design of catalytically competent conformations.

International cooperations

Noelia Ferruz, Centre for Genomic Regulation (CRG), Barcelona, Spain

Research projects

BioTechMed YRG: “Active site transfer to generate new enzymes”

FWF PAT1730825: “Yield enhancement of recombinant enzyme production

Talks at national and international conferences/meetings

1. Kolathuparambil, S.: *Computational design of new proteases*, FEBS Advanced Practical and Lecture Course on Computational Approaches to Understanding and Engineering Enzyme Catalysis, Stockholm, Sweden, September 2025
2. Kolathuparambil, S.: *Computational design of new proteases*, European RosettaCon 2025: Designing Functional Proteins for the Future, Ljubljana, Slovenia, October 2025
3. Lechner H.: *Protein Design with ProtFlow*, EMBO Practical Course - AI for protein design, Puerto Varas, Chile, November 2025

Publications:

1. Braun, M., Tripp, A., Chakatok, M. *et al*. *Computational enzyme design by catalytic motif scaffolding* (2026) ***Nature*** 649, 237–245, DOI: 10.1038/s41586-025-09747-9