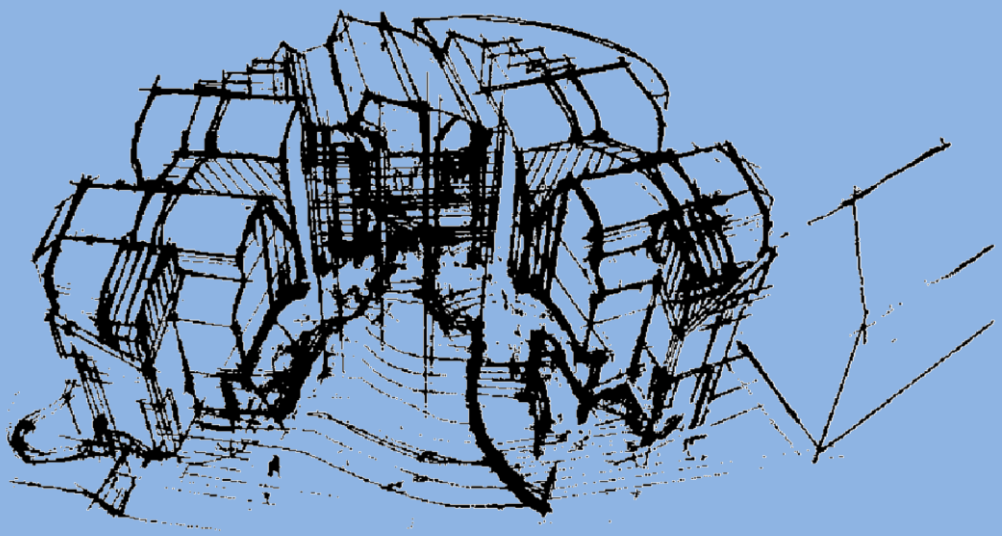
Graz University of Technology
Austria

Institute of Biochemistry



Annual Report 2024

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

Highlights of 2024

The Institute is now also present on various social media channels! Check out recent updates and highlights by following the QR codes below:

The homepage has gone through an intense updating phase – thanks to Christine Rother for all her efforts. 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:

2024 was an exciting year for the Macheroux group. Stefanie Baldauf, Aleksandar Bijelic and Katharina Fuchs took part in the Flavins and Flavoprotein Symposium 2024 in Atlanta. As you can see in the picture on the left they enjoyed their time at the conference, especially at the concluding banquet at the Fernbank Museum in Atlanta, GA, USA.
Another highlight was the yearly retreat in Southern Styria, where the whole group could enjoy some nice days at a traditional Buschenschank with hiking, excellent food and wine of the region.

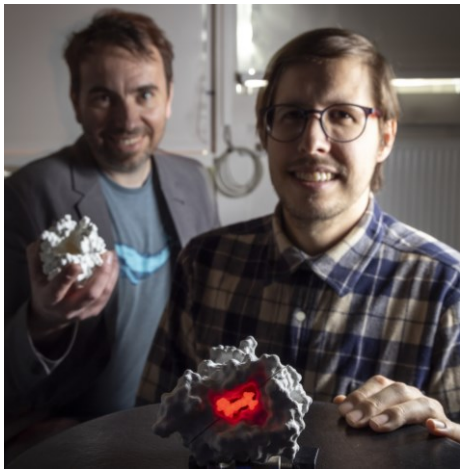
In September Pedro Freitas from the Univesity of Madeira joined the group of Peter Macheroux to work on a collaborative project and to deepen his practical knowledge in biochemistry.

Winkler group: In 2024 the group of **Andreas Winkler (AGW)** was not only engraving its initials into pumpkins, but also very active in communicating its research activities at various international meetings. For example, Ursula Vide organized the *Gordon Research Seminar on Photosensory Receptors and Signal Transduction* in Lucca (Italy). Another highlight was the review article on "*Phytochrome Structure and Function*" published in the *Annual Review in Plant Biology* series – a major joint effort by Jon Hughes and Andreas Winkler. 2024 also marked the finishing year for three PhD students in the group (Ursula Vide, Massio Totaro and Hieu Tran); while we were happy to see them successfully graduating, this also meant major restructuring within the group. However, several Master students and new arrivals in 2025 have kept the group up and running. Collaborations on using the dedicated mass spectrometry infrastructure for advanced protein analytics (intact mass, native mass and hydrogen-deuterium exchange experiments) were also steadily increasing and kept Philipp Pelzmann occupied with their respective analyses.

berdorfer group:

2024 held a lot of highlights for the Oberdorfer lab. Projects were finished with two PhD students finishing their dissertation and a successfully defended master thesis. Several manuscripts were uploaded to preprint servers and will hopefully be fully published soon. This year also marked the highest number of public lectures given by group members, which had a tremendous impact on international visibility for the group. Lastly, the clear highlight of 2024 was the Nobel Prize in Chemistry being awarded in part to computational protein design and the possibility for Gustav Oberdorfer to celebrate with the Nobel Laureates during the award ceremony in Stockholm.

echner group: Horst Lechner and his PhD student Sajith Kolathuparambil are progressing their work, to design new proteases. They established a *S. cerevisiae* based FACS screening system in collaboration with Tobias Eisenberg (University of Graz) and looking forward to test their designs. Claudia Hrastnik joined the group as technician after the retirement of Michael Murkovic. Ahmed Alagic was employed for 3 months via a „Anschubfinanzierung“ aiming to increase the yields of heme-dependent enzymes. Additionally, in collaboration with the Macheroux group, an ERASMUS student (Pedro Almeida Freitas from Madeira) is working on the characterization of computationally design variants of BBEs to improve their production.



urkovic group: The Functional Food group with its focus on the use of food wastes for cultivation of edible fungi and the development of food ingredients on basis of microalgae came to an end with the retirement of Michael Murkovic in 2024. Starting to study technical chemistry at Graz University of Technology in 1979 he spent 45 years of his life in the realm of chemistry and, in particular, food chemistry in Graz. Reflected in several research stays and teaching duties all over the world, Michael was very active in research, but also in teaching, where he also shaped the development of curricula for hundreds of students over the years. As far as PhD students are concerned, he accompanied 14 young researchers on their way to scientific independence. We all wish Michael the best for upcoming challenges in his retirement phase with plenty of time to invest in his hobbies – traveling and sustainable cultivation of food.

Biochemistry Group

Group leader: Peter Macheroux
Secretary: Christine Rother
Senior research scientist: Silvia Wallner
Postdoctoral fellow: Aleksandar Bijelic
PhD students: Stefanie Baldauf, Katharina Fuchs, Bianca Kerschbaumer
Master students: Anja Lex, Hannah Gasser, Barbara Millonig
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 (thesis project of Stefanie Baldauf supported by Dr. Silvia Wallner).

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

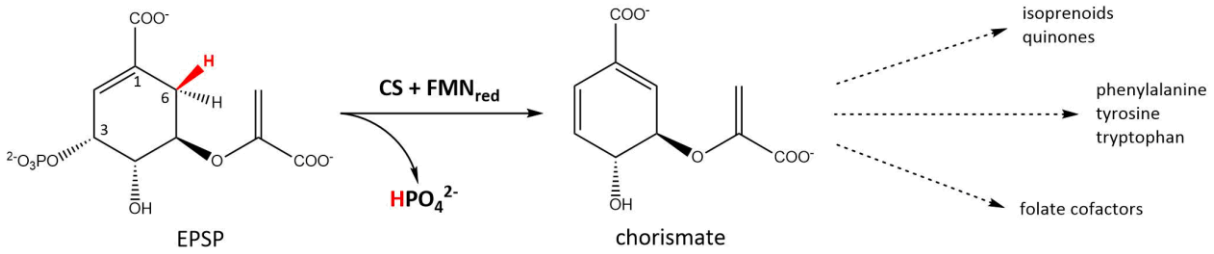
Chorismate synthase as a potential target for pharmaceutically useful inhibitors

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 Katharina Fuchs and master project of Barbara Millonig; supported by Dr. 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.

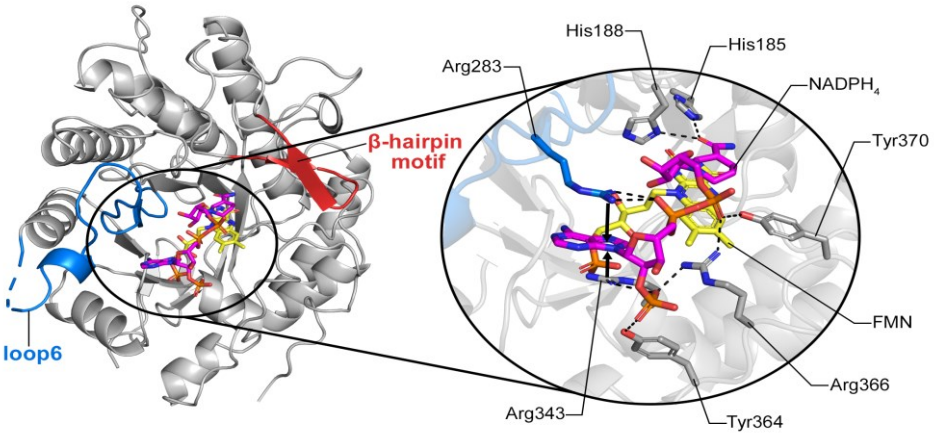
[3] Bueno *et al*. *New inhibitors of chorismate synthase present antifungal activity against Paracoccidioides brasiliensis* (2019) ***Future Microbiol.*** 14:969-980.

Ene-reductases: shifting the cofactor preference of *Sl*OPR3 from NADPH toward NADH

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 (thesis project of Bianca Kerschbaumer). 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 nicotinamide group of the cofactors binds in the same way (i.e., above the flavin), their (2'-phosphate)-adenosine tails bind to different sites within the active site cavity [4]. This structural information is currently being leveraged to design *Sl*OPR3 variants with switched cofactor preferences.



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).

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 (Kerschbaumer *et al.*, 2024). 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*.: *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

Doctoral Thesis completed

Bianca Kerschbaumer: *Biochemical and structural studies on selected flavoenzymes.*

Flavoproteins are an amazingly diverse class of enzymes characterized by their flavin cofactors, including flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). These cofactors can be non-covalently, monocovalently, or bicovalently attached to the protein. Among them, berberine bridge enzyme (BBE)-like enzymes and ene-reductases have attracted considerable interest due to their unique catalytic function and versatile applications.

Although BBE-like enzymes are abundant in plants, bacteria, and fungi, their heterologous production and subsequent characterization pose significant challenges. As a result, their functions remain largely elusive. Therefore, four fungal BBE-like enzymes (i.e., EasE from *Aspergillus japonicus*, CnsA from *Penicillium expansum*, and NcBBE10 and 12 from *Neurospora crassa*) were selected to broaden the understanding of this protein family, especially regarding their heterologous production. EasE and CnsA were produced in small amounts using a cell-free insect expression system. NcBBE10 and 12 could be detected using secreting *Komagataella phaffii* strains. Further, this work resulted in the development of a more accurate and convenient screening method for secreting K. phaffii strains, which will simplify future screening procedures.

Ene-reductases catalyze the asymmetric reduction of C=C double bonds and thus provide an environmentally friendly solution for synthesizing chiral compounds. However, their dependence on nicotinamide coenzymes (NAD(P)H) and the widespread preference of most ene-reductases for the more expensive and less stable NADPH over NADH pose significant challenges in industrial applications. Despite extensive research on ene-reductases over the past decade, the underlying mechanisms for their nicotinamide coenzyme preference remain poorly understood. Therefore, 12-oxophytodienoic acid reductase 3 from *Solanum lycopersicum* (SlOPR3), a NADPH-preferring ene-reductase of the Old Yellow Enzyme family, was chosen as model enzyme to study this fundamental issue.

To evaluate the coenzyme specificity and differences in the binding modes between NADPH and NADH to SlOPR3, a variety of experimental techniques such as bioinformatics (including molecular docking and molecular dynamics simulations), mutagenesis, stopped-flow rapid kinetics, and X-ray crystallography were employed. The findings revealed two structural hotspots, loop 6 and the arginine triad comprising R283, R343, and R366, along with the β-hairpin flap (BHP). Loop 6 and the BHP are primarily involved in nicotinamide coenzyme binding and stabilization to ensure proper H-transfer from the nicotinamide moiety to the isoalloxazine ring and play a complementary role by providing anchor sites for the tails of NADPH and NADH, respectively.

Master Theses completed

Hannah Gasser: *Switching the coenzyme specificity of morphinone reductase from Pseudomonas putida from NADH to NADPH.*

Ene-reductases (ERs) are important biocatalysts that catalyze the asymmetric reduction of activated C=C bonds at the expense of nicotinamide coenzymes (NAD(P)H). The most investigated ERs belong to the flavin-dependent Old Yellow Enzyme Family (OYE) of oxidoreductases, which strongly prefer NADPH or NADH as a hydride donor. Even though OYEs have been extensively studied regarding their catalytic mechanism, a deeper understanding of the coenzyme binding mechanism and coenzyme specificity in OYEs is still missing. A previous study on the NADPH-preferring 12-oxophytodienoic acid reductase 3 from *Solanum lycopersicum* (*Sl*OPR3) indicated that loop 6 and the β-hairpin (connecting α-helix 3 and β-strand 3) are primarily involved in NADPH and NADH binding by ERs, respectively.

In this work, the NADH-dependent flavoenzyme morphinone reductase (MR) from Pseudomonas putida (*Pp*MR) was chosen as a model enzyme to further investigate this hypothesis and eventually switch its nicotinamide specificity from NADH toward NADPH. Different *E. coli* strains were tested to optimize the heterologous protein production of *Pp*MR and the strain *E. coli* OverExpressTM C43 (DE3) was identified as the best expression strain for producing *Pp*MR based on protein yield, functionality, and thermal stability.

In a semi-rational approach, loop 6 and the β-hairpin of wild-type MR (MRwt) were targeted and swapped with the corresponding regions of the NADPH-preferring cyanobacterial ene-reductase 1 from Nostoc sp. PCC7120 (NostocER1) and SlOPR3. Four single-swap MR variants and two double-swap MR variants were produced in *E. coli* OverExpressTM C43 (DE3), purified, and characterized. Pre-steady state kinetics of *Pp*MRwt and MR variants with NAD(P)H revealed a biphasic reductive half-reaction, with a faster rate corresponding to flavin reduction at 464.5 nm. Pre- steady state kinetics showed that the cofactor preference of *Pp*MR could be successfully switched from NADH to NADPH by swapping loop 6 (L6) and the β-hairpin (HP) flap with the corresponding regions of *Sl*OPR3. The catalytic efficiency k_{eff} (k_{red}/K_d) of MR-L6HPOPR3 with NADPH is two times higher than that with NADH. Surprisingly, incorporating only loop 6 from SlOPR3 into *Pp*MR led to an increased NADH-affinity (K_d = 102 ± 16 μM) compared with that of *Pp*MRwt (K_d = 260 ± 40 μM), yielding a four-times higher k_{eff} than that for *Pp*MRwt. These results suggest that loop 6 influences not only the affinity of *Pp*MR toward NADPH but also toward NADH.

In conclusion, loop swapping proved to be an efficient approach for switching the cofactor specificity in *Pp*MR. In order to gain deeper insights and a better understanding of the cofactor binding mechanism in OYEs, further structural elucidation will be imperative in future studies.

Anja Lex: *Interaction of hETF wildtype and variants with client dehydrogenases and its natural electron acceptor hETF-QO*

The respiratory chain in mitochondria is essential for the survival of cells in many organisms, such as humans. Electron-transferring flavoproteins (ETFs) play an essential role in the conversion of energy from food into chemical energy in the form of ATP. ETF accepts electrons from various flavin-dependent dehydrogenases and transfers them to the membrane-

bound flavoprotein ETF-QO, which passes the electrons on to the ubiquinone pool. For this transfer, ETF must be present in its open conformation. It is known that the absence of a salt bridge, which is formed between the amino acids αN259 and βE165, favors the open form. Previous studies by Drs. Peter Augustin, Marina Toplak and Sami Ullah Khan have revealed that the flavin cofactor from human ETF (hETF) can modify pH-dependently and irreversibly, resulting in an oxidized form of flavin with an 8-formyl group (8f-FAD). It was observed that this formylation proceeds differently when comparing the wildtype with the two variants hETF αN259A and βE165A.

Here, the hypothesis was developed that the oxidation of the 8α-methyl-group of FAD can be prevented by interaction of hETF with a client dehydrogenase. In this context, it is assumed that the dehydrogenase spatially shields the cofactor and, thus, protects it from oxidation. Spectrophotometric measurements were used to test this hypothesis, whereby hETF was examined in the presence and absence of selected dehydrogenases, i.e. human medium-chain acyl-CoA and isobutyryl-CoA dehydrogenase. These measurements did not confirm the hypothesis. However, differences in the modification between the variants could be observed.

In a further part of the work, an attempt was made to produce the membrane-bound hETF-QO, which acts as an electron acceptor of hETF, as a soluble and fully functional protein by heterologous expression in E. coli as well as to investigate the interaction with hETF. However, the production of hETF-QO was unsuccessful with the available strains and methods. Future efforts with other production strains as well as other methods may lead to the successful generation of soluble hETF-QO.

International cooperations

Jörg Hartig & Malte Sinn, University of Konstanz, Germany

Francesco Mutti & Tanja Knaus, University of Amsterdam, The Netherlands

Research projects

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

FWF DOC46: "CATALOX – Catalytic applications of oxidoreductases; doc.funds"

Publications

1. Carmona, G.O., Lahham, M., Poliak, P., Goj, D., Friesser, E., Wallner, S., Macheroux, P., Oostenbrink, C.: *Understanding the riddle of amine oxidase flavoenzyme reactivity on the stereoisomers of N-methyl-dopa and N-methyl-tyrosine* (2024) ***Mol. Recognit.***, 37, e3068. DOI: 10.1002/jmr.3068

2. Kerschbaumer, B., Totaro, M.G., Friess, M., Breinbauer, R., Bijelic, A., Macheroux, P.: *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

3. Knaus, T., Macheroux, P., Mutti, F.G.: *Fus-SMO: Kinetics, biochemical characterization and in silico modelling of a chimeric styrene monooxygenase demonstrating quantitative*

coupling efficiency (2024) ***ChemBioChem***, 25, e202300833. DOI: 10.1002/cbic.202300833

4. Friess, M., Sahrawat, A. S., Kerschbaumer, B., Wallner, S., Gomez, A. T., Torvisco, A., Fischer, R., Gruber, K., Macheroux, P., Breinbauer, R.: *Asymmetric synthesis of chiral 2-cyclohexenones with quarternary stereocenters via ene-reductase catalyzed desymmetrization of 2,5-dicyclohexadeinones* (2024) ***ACS Catal.***, 14, 7256-7266. DOI: 10.1021/acscatal.4c00276

5. Kerschbaumer, B., Macheroux, P., Bijelic, A.: *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

6. Bijelic, A., Macheroux, P.: *Structure of human phospholipase D3, a single-strand exonuclease associated with Alzheimer's disease* (commentary) (2024) ***FEBS J.***, 291, 5394-5397. DOI:10.1111/febs.17319

7. Fuchs, K, Totaro, M.G., Toplak, M., Bijelic, A., Macheroux, P.: *Investigation of the inhibitory properties of azo-dyes on chorismate synthase from Paracoccidoides brasiliensis* (2024) ***J. Enz. Inhib. Med. Chem.***, 39, 1, 2427175. DOI:10.1080/14756366.2024.2427175

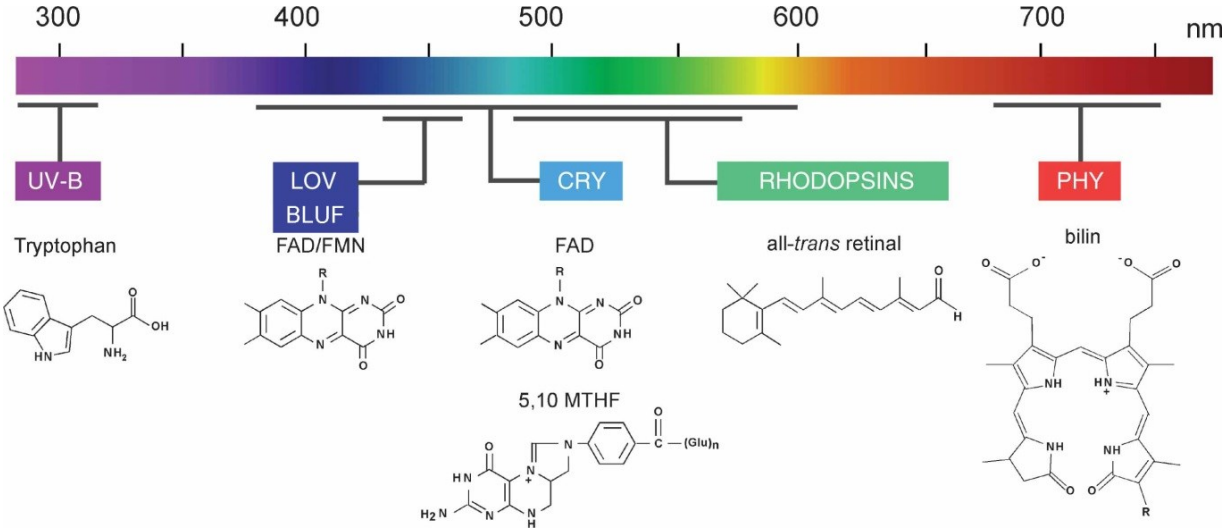
8. Shah, K., Kracher, D., Macheroux, P., Wallner, S., Pick, A., Kourist, R.: *Discovery and characterization of NADH oxidases for selective sustainable synthesis of 5-hydroxymethylfuran carboxylic acid* (2025) ***J. Biotechn.***, 398, 18-28. DOI:10.1016/j.jbiotec.2024.11.009

Photoreceptor Group

Group leader: Andreas Winkler
PhD students: Oliver Eder, Maximilian Fuchs, Julia Schwekendiek, Massimo Totaro (joint with AGO), Hieu Tran, Uršula Vide
Master students: Miriam Huber, Gabriela Schickle
Bachelor student: Stefan Minnich
Technical staff: Philipp Pelzmann, Sophie Zweytick
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.

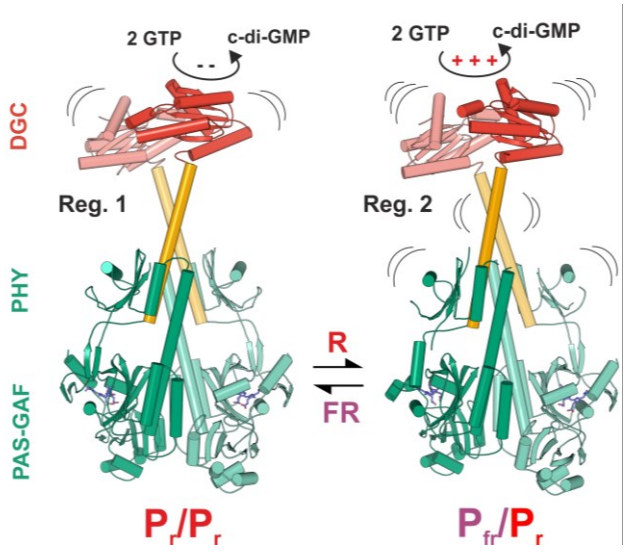


Overview of photoreceptor families and their corresponding cofactors covering the UV/Vis range of the electromagnetic spectrum (adapted from Heintzen WIREs Membrane Transport and Signaling 2012, 1:411–432).

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 they correlate with non-canonical spectroscopic signatures (only partial Pfr formation) in these systems was the focus of the PhD thesis of Hieu Tran and Oliver Eder recently also followed up on this by characterizing a close homolog of *Is*PadC using cryo-EM in collaboration with Elmar Behrmann (University of Cologne).

In an attempt to characterize molecular determinants of asymmetry in *Is*PadC, Hieu Tran attempted to use NMR for looking into the conformational dynamics of these complex dimeric systems. Initial analyses using Methyl-TROSY by labelling with ^{13}C isoleucine and methionine did not show significant shifts in NMR spectra due to the limited activation from a fiber-coupled LED via top-down illumination. While roughened optic fibers resolved the issue of low activation, the propensity of *Is*PadC to transiently form tetrameric assemblies via an "artificial interface" caused severe losses in signal intensity and, thereby, stopped efforts to follow up on asymmetric properties of light activation via NMR. Other homologs lacking the artificial interface, might be interesting for follow up NMR studies, but the focus is now on cryo-EM.

In the absence of structural data, Hieu Tran eventually shifted his focus on specific variants of *Is*PadC that showed interesting properties with respect to stabilizing either the Pr or the Pfr state. In conclusion, he could show that *Is*PadC still features photostationary states with only partial Pfr formation in variants that have extremely long Pfr lifetimes. While rather indirect, these results are in-line with preliminary cryo-EM data and support a structural asymmetry where the two protomers influence each other upon light activation [3].

Since the residues addressed by Hieu Tran were all first shell residues surrounding the cofactor, which are mostly conserved throughout bacteriophytochromes, but still 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 [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] Tran *et al*.: *Dynamics-driven allosteric stimulation of diguanylate cyclase activity in a red light-regulated phytochrome* (2024) ***J. Biol. Chem.***, 300, 5, 107217

[4] Eder *et al*.: *Integrating Protein Sequence Design and Evolutionary Sequence Conservation to Uncover Spectral Tuning Sites in Red-Light Photoreceptors* (2025) ***Sneak Peak Preprint***, https://dx.doi.org/10.2139/ssrn.5197472

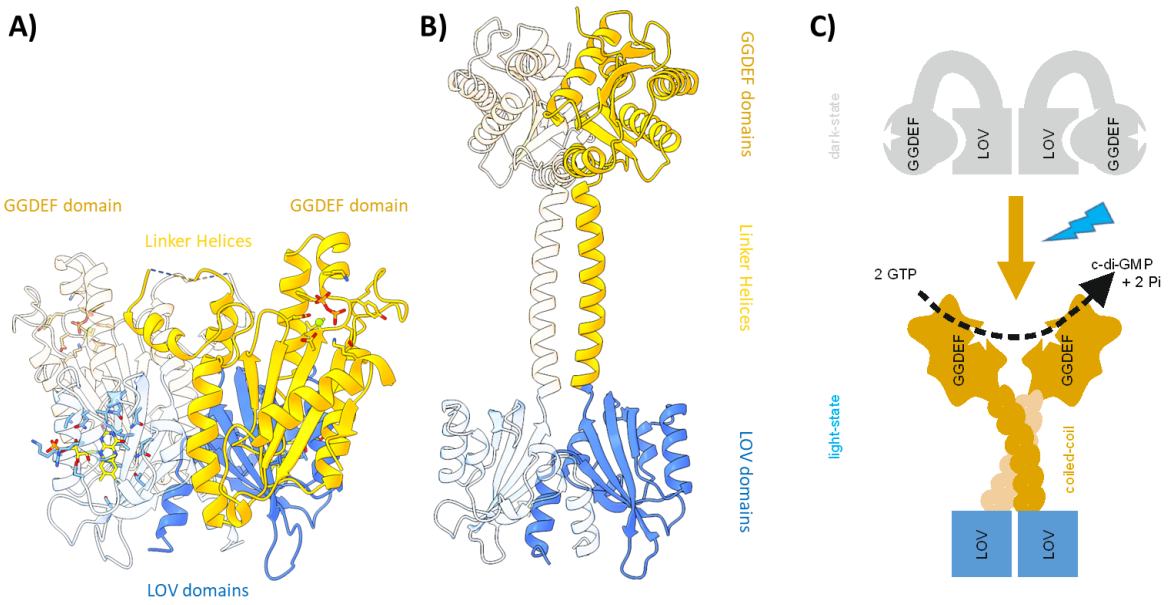
Blue-light-regulated diguanylate cyclases

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 [5] is central to this function. In addition, LOV domains also have a high potential in applied biosciences, where they contribute significantly to the field of optogenetics using a variety of LOV-coupled effector domains [6].

In order to complement our efforts in understanding the modularity of sensors regulating diguanylate cyclases described above, we also work on LOV-regulated GGDEF domains (PhD projects of Uršula Vide and Maximilian Fuchs). To elucidate molecular mechanisms of light regulation we are studying naturally occurring LOV-GGDEF systems and integrate 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 lies 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 [7].

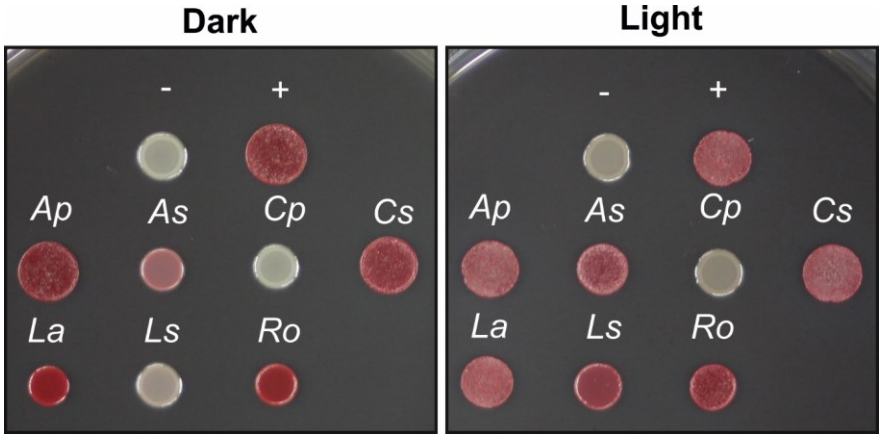
Additional aims of the PhD thesis of Uršula Vide (with the support of Gabriela Shickle) went in the direction of addressing the light activated state on a molecular level and using this system for optogenetics and other applications that require precise temporal and spatial control of enzymatic activity or other biological outputs. Besides focusing on the role of specific residues in the sensor-effector linker and at the extensive inhibitory interface between the two domains, also eliminating feedback product inhibition was investigated after testing a range of amino acid substitutions based on a computational tool developed by Massimo G. Totaro [8].



Basis for the LOV-activated diguanylate cyclase switch mechanism. Crystal structure (**A**) of a LOV-GGDEF homolog and its predicted AlphaFold model (**B**) showing a coiled-coil sensor-effector linker. The dark, inhibited conformation (**A**) is characterized by a tight association of the sensory LOV and catalytic GGDEF domains in the dimeric assembly, which prevents the productive encounter of GGDEF domains as required for catalysis. The efficient caging of the effector domains is only released upon structural rearrangements induced by blue light illumination (**C**).

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 can be 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. This could result in complex molecular logics that affect the regulation of the effector's active or inactive conformations.

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 producing and purifying diverse proteins. The screening shows distinct changes in diguanylate cyclase activity between dark and light states, which match changes observed in *in vitro* experiments. In addition to our RecLadC homologs, we can coproduce possible cognate kinases, assessing their potential to modify the activity of the effector domain. Since in vitro phosphorylation of our model system appears feasible only for the isolated Rec domain, this approach seems most promising when it comes to understanding the function of the receiver domain.



***In vitro* activity of different dual-sensor diguanylate cyclases.** The screening showed distinct differences in activity when plates were illuminated with blue light during the screening process. The changes are especially pronounced for *As* and *Ls*RecLadC

Only few dual-sensor systems were characterized in detail so far and, therefore, understanding their regulation capacities in detail remains to be uncovered. Our efforts to decipher the molecular mechanisms of signal processing in these proteins are also aided by structural data obtained through X-ray crystallography. Promising preliminary data has been obtained for Rec-only and Rec-LOV truncation constructs. Insights into cooperative domain interplay could eventually facilitate the development of molecular tools for implementing additional regulatory networks in optogenetics.

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

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

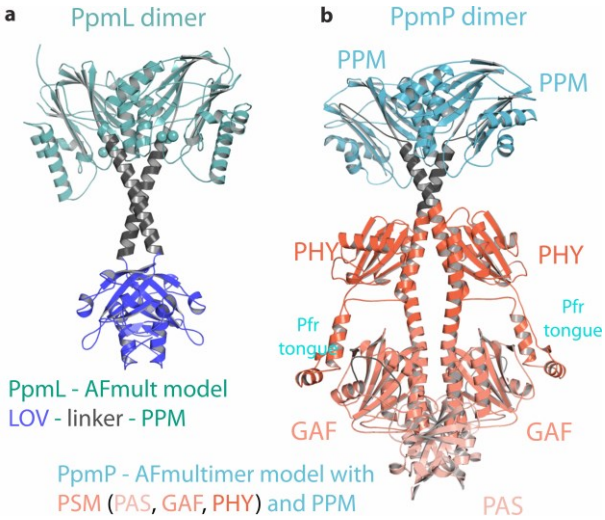
[7] Vide *et al.*: *Illuminating the inner workings of a natural protein switch: Blue-light sensing in LOV-activated diguanylate cyclases* (2023) ***Sci. Adv.***, 9, 31, eadh4721

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

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, Sophie Zweytick and Miriam Huber) 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.

While still rather preliminary data, enzymatic characterization of both systems with the phosphatase substrate analog para-nitrophenyl phosphate (pNPP) indicate light regulation capacities in both photoreceptor-PPM families that we term PpmP and PpmL for PPM phosphatase phytochrome and LOV, respectively. 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.

Doctoral Theses completed

Hieu Tran: *Analysis of structural asymmetry in red/far-red light-activated bacteriophytochromes*

Using light as a trigger to initiate central biological processes, phytochromes have been adapted and evolved in plants, algae, fungi, and bacteria. Due to the deep tissue penetration of red light and their reversible photoactivation, red/far-red light-activatable phytochromes hold great potential as non-opsin-based optogenetic tools (OTs). One such example is a prototypical red light-activated bacteriophytochrome, *Idiomarina species* A28L phytochrome-activated diguanylyl cyclase – *Is*PadC, which exhibits some unique properties. It transitions between red-absorbing (Pr) and far-red-absorbing states (Pfr) following a prototypical phytochrome including chromophore isomerization, reorientation of residue's sidechains proximal to the binding pocket as well as the refolding of the PHY-tongue and the N-terminal segment. The proposed active conformation of *Is*PadC is suggested to be an asymmetric heterodimer structure in which Pr/Pfr coexist. This asymmetry is supported by *Is*PadC's non-canonical spectral characteristics and is most likely due to the variety of amino acids in the proximity of the cofactor that allows systems to adapt to the needs of an organism during evolution.

I tackle providing confirmation of the above hypotheses using integrative structure biology methods based on a known crystal structure of *Is*PadC and its proposed active conformation *Is*PadC^{Reg2}. The structure asymmetry and related signal transduction were addressed using methyl-TROSY NMR on the photosensory module including the coiled-coil linker (PSMcc) of *Is*PadC, and additional insights were gained through HDX-MS and other biochemical characterizations on IsPadC variants, created from single amino acid substitutions

targeting the PHY-tongue, cofactor binding site and DIP motif. They suggested that functional responses are driven by conformational dynamics rather than just the equilibrium between dark- and light-adapted states. Furthermore, I present an attempt to use helix mimetics to restore the typical spectral and structural characteristics of wild-type *Is*PadC in an *Is*PadC variant lacking the PHY-tongue. However, this idea faced many difficulties that need further improvement. In addition, crystallization attempts were done to address the mechanism of tetramerization upon illumination of a novel OT – iLight, which was derived from the photosensory module of *Is*PadC. While the structure of iLight crystalized in the dark requires higher resolution data, the light state structure remains completely elusive.

Ursula Vide: *Molecular Mechanisms of Light Regulation in LOV-Diguanylate Cyclases*

Organisms have evolved complex regulatory systems that integrate multiple pathways to maintain homeostasis and ensure survival. These systems respond to external stimuli, such as light, by activating sensor and effector modules to counteract potential disruptions. Photoreceptors are an integral part of these systems, as they interpret light signals to help organisms adapt their behavior and physiology. Among these, the flavin-dependent light-oxygen-voltage (LOV) domain family received considerable attention in the past few decades. These compact protein domains, typically around 110 amino acids, are found in a wide range of organisms, including plants, fungi, algae, and pathogenic bacteria.

A common effector domain linked to LOV domains is the GGDEF diguanylate cyclase (DGC), which synthesizes the bacterial second messenger cyclic dimeric guanosine monophosphate (c-di-GMP). This dissertation investigated the molecular mechanisms underlying light regulation in bacterial LOV-activated diguanylate cyclases (LadCs), focusing on the structural changes induced by blue light and how these changes propagate to the DGC domains.

Through an integrative structural biology approach, this study identified a two-stage activation mechanism in long-linker LadC homologs. They function as natural DGC switches and are promising candidates for optogenetic applications. In the dark state, their activity is inhibited by caging the DGC effector domains to the dimeric LOV core, while illumination stimulates DGC activity through the release of effector domains and the formation of an extended coiled coil. Structural insights from the dark-state crystal structure of one long-linker LadC homolog guided the design of substitutions targeting residues at the inhibitory interface between LOV and DGC effector domains, as well as specific positions in the linker helix. The findings from various variants highlighted the central role of the linker helix in signal transmission, a recurring motif in modular proteins. This work contributes a valuable addition to the optogenetic toolbox, as well as strategies for performance enhancement of the LadC switch.

Massimo Totaro: *Bridging physics-based simulations and experimental approaches in bacteriophytochrome protein–protein interface engineering*

Joint project - Abstract included in the respective section of the Protein Design group

Master Thesis completed

Gabriela Shickle: *Molecular Mechanisms of Signal Integration in blue light-regulated Diguanylate Cyclases*

Unlocking the secrets of bacterial communication through c-di-GMP signaling has led researchers to investigate the intricate molecular mechanisms of light regulation in LOV-diguanylate cyclases. This master thesis delves into the fascinating world of *Ms*LadC and other LOV-homologs, exploring the hypothesis that amino acid substitutions in the linker region can either stabilize or inhibit its enzymatic function, unveiling a two-switch regulation mechanism.

Drawing from previous studies and using intentionally designed variants targeting the linker heptad repeats of *Ms*LadC, this research confirms the pivotal role of linker helices in modulating DGC activity. Substituting amino acids in the linker region either significantly boosts or eliminates DGC activity, shedding light on the complexity of the regulatory mechanisms at play. Challenges arise in isolating and purifying the protein, as certain variants display poor solubility and aggregation tendencies. Despite these hurdles, the study uncovers intriguing insights into the structural dynamics of DGCs and their regulatory mechanisms, offering clues to the intricacies of bacterial signaling pathways. Notable differences emerge between *in vivo* and *in vitro* DGC activities, showcasing the impact of reaction environments on enzyme functionality. As the research unfolds, the promising potential of *Ms*LadC in optogenetic applications becomes evident, further emphasizing the significance of understanding the molecular mechanisms of light regulation in LOV-Diguanylate cyclases. The observed variations underscore the importance of considering the context in which enzymes are studied, paving the way for potential applications of *Ms*LadC in various biological systems.

International cooperations

Elmar Behrmann, University of Cologne, Cologne, Germany

Jon Hughes, Justus-Liebig-University Giessen, Giessen, Germany

Igor Schapiro, TU Dortmund, Dortmund, Germany

Research projects

FWF P32022: “Signal Integration in Phytochrome-linked Diguanylate Cyclases”

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

FWF DOC130: “BioMolStruct – Biomolecular Structures and Interactions; doc.funds”

Organization of conferences/meetings

1. Oberdorfer, G. and Winkler A..: Organizers of the *Frontiers in Integrative Structural Biology and Biophysics Symposium*, Graz, Austria, December 2024.

2. Vide, U..: Organizer and Chair of the *2024 Gordon Research Seminar on Photosensory Receptors and Signal Transduction*. Lucca, Italy, March 2024

Talks at national and international conferences/meetings

1. Fuchs, M.: *Finding LOV: In crystallo optical spectroscopy of a dual sensor photoreceptor.* Frontiers in Integrative Structural Biology and Biophysics, Graz, Austria, December 2024.
2. Winkler, A.: Towards dynamics-driven allosteric regulation in modular sensor-effector systems. University of Graz Chemistry PhD Seminar, Graz, Austria November 2024
3. Winkler, A.: *Addressing sensor-effector modularity in light-regulated diguanylate cyclases*. Seminar at City University of New York, New York, U.S.A., September 2024
4. Winkler, A.: *The intricate molecular machinery of a blue light switch*. 4th Optogenetic Technologies and Applications Conference, Boston, U.S.A, September 2024
5. Winkler, A.: Addressing sensor-effector modularity in light-regulated diguanylate cyclases. Max Planck Institute for Medical Research, BMM Retreat 2024, Günzburg, Germany, July 2024
6. Winkler, A.: *Towards dynamics-driven allosteric regulation in modular sensor-effector systems*. TCVB Science Day 2024, Graz, Austria, June 2024
7. Eder, O.: *Bilin D-ring properties: Lessons from a Bacteriophytochrome binding PCB.* 3rd Scientific Retreat - BioMolStruct, Seggau, Austria, June 2024.
8. Fuchs, M.: *Finding LOV: Structural studies of a dual sensor diguanylate cyclase.* 3rd Scientific Retreat - BioMolStruct, Seggau, Austria, June 2024.
9. Eder, O.: *Bilin D-ring properties: Lessons from a Bacteriophytochrome binding PCB*. 2024 Gordon Research Conference on Photosensory Receptors and Signal Transduction: Light-Dependent Molecular Mechanism, Cellular Response and Organismal Behavior. Lucca, Italy, March 2024.
10. Vide, U.: Blue light-regulated LOV-diguanylate cyclases: Highly efficient molecular switches. 30th Doc-Day of the NAWI Graz Doctoral School of Molecular Biosciences and Biotechnology. Graz, Austria, February 2024
11. Fuchs, M.: *Influence of oligomeric state on Protein Select™.* ÄKTA™ User Days 2024, Munich, Germany, April 2024.

Publications

1. Hughes, J., & Winkler, A.: *New insight into phytochromes: Connecting structure to function* (2024) ***Annu. Rev. Plant. Biol.***, 75 (1), 153-183*,* DOI: 10.1146/annurev-arplant-070623-110636
2. Totaro M., Vide, U., Zausinger, R., Winkler, A., Oberdorfer, G.: *ESM-Scan-a tool to guide amino acid substitutions* (2024) ***Prot. Sci.***, 33, 12, e522. DOI: 10.1002/pro.5221
3. Tran, H., Eder, O.M., Winkler A.: *Dynamics-driven allosteric stimulation of diguanylate cyclase activity in a red light regulated phytochrome* (2024) ***J. Biol. Chem.***, 300, 5, 107217. DOI: 10.1016/j.jbc.2024.107217
4. 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) ***Sneak Peak Preprint***, DOI: 10.2139/ssrn.5197472

Protein Design Group

Group leader:	Gustav Oberdorfer
PhD students:	Markus Braun, Wael Elaily, Julia Messenlehner, Anna Schrüfer, Massimo Totaro, Adrian Tripp, Florian Wieser
Master students:	Melanie Moser, Sigrid Kaltenbrunner, Andreas Zechner, Johannes Peterlechner
Bachelor students:	Anna Hirschberger, Antonia Suanjak
Technical Staff:	Alma Makic, David Stoll, Morakot Chakatok, Birgit Grill
Visiting students:	Martin Oderbas (BTM Lab Rotation), Jelena Ożegović (Erasmus Plus), Shlomo Yakir Hoch (exchange PhD student Weizmann Institute of Science),
Visiting professors:	Allon Hochbaum (University of California Irvine)

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.

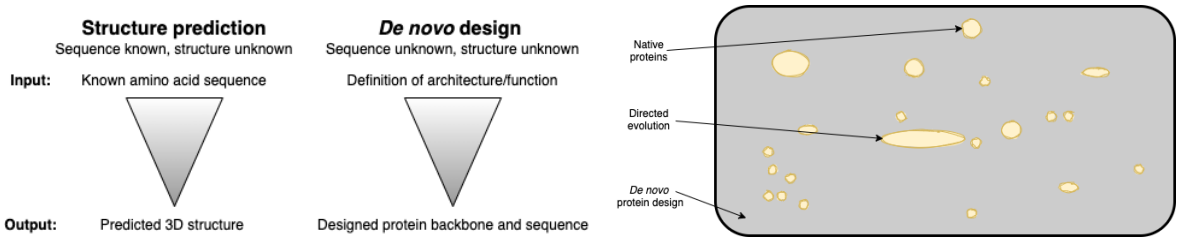


Figure (left) *Differences in structure prediction and de novo protein design* ***(right)*** *Illustrative representation of protein sequence space (grey). Sequence analysis of native proteins shows that tight clusters of protein families can be identified (beige).*

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.

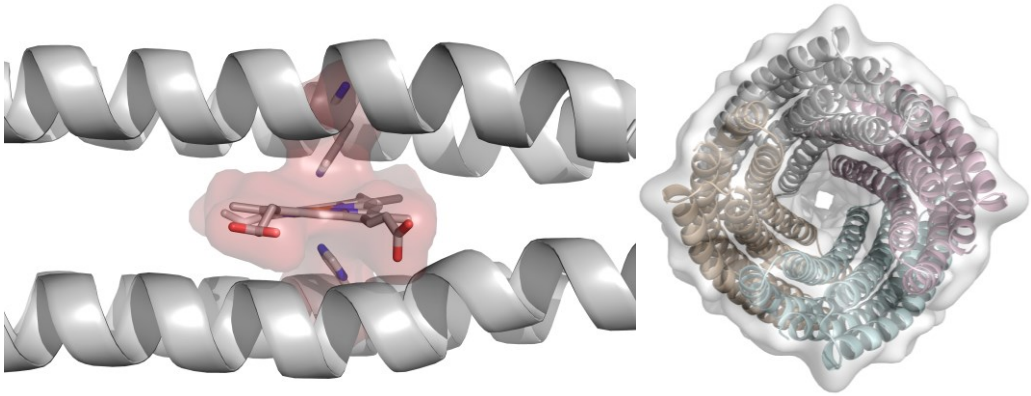


Figure 2. (left) *Computationally designed 4-helix bundle with a designed binding site for b-type heme. It can clearly be seen how much the otherwise straight helices had to be bent, to accommodate the heme cofactor.* ***(right)*** *top view of a computationally designed 20-helix bundle. This is a completely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

Master Thesis completed

Sigrid Kaltenbrunner: *Design and Characterization of de novo Enzymes and de novo Protein Binders*

De novo protein design represents a ground-breaking approach to engineering proteins with tailor-made properties, enabling the creation of enzymes to catalyze in principle any chemical reaction, or proteins that precisely target therapeutic molecules such as cancer biomarkers. Unlike natural proteins, which are constrained by evolution, de novo designs can be optimized for enhanced stability, specificity, and efficiency, including thermostability, solubility, or resilience under harsh industrial conditions. This ability to design proteins from scratch offers immense potential for biotechnology, medicine, and materials science, opening doors to innovations such as biocatalysts for green chemistry, custom therapeutics, and novel biomaterials. In this thesis, I present the application of de novo protein design to create protein binders, enzymes, and their biophysical, biochemical, and structural characterization.

In Part I, the design of a de novo protein binder targeting the human epidermal growth factor receptor (EGFR), a critical biomarker in various cancers, is detailed. This project, conducted in collaboration with Adrian Tripp as part of the Adaptyv Bio Protein Design Competition, utilized the “ProtFlow” Python package. This tool integrates multiple protein design methods, including RFDiffusion, Rosetta, LigandMPNN, ESMFold, and AlphaFold, into a streamlined workflow. Out of over 700 submitted designs, our de novo binder achieved third place in the competition, demonstrating high-affinity binding with a dissociation constant (KD) of $(2.3 \pm 0.8) \times 10{-}5$ M.

Part II highlights the application of RiffDiff [1], a machine learning and atomistic modeling strategy, to design 18 *de novo* enzymes for the Morita-Baylis-Hillman reaction. This part provides a detailed account of the iterative design process. Experimental evaluation revealed that at least five of the designed enzymes successfully catalyzed the first step of the reaction, underscoring the successful creation of accessible binding pockets and a high degree of active site preorganization.

Finally, Part III focuses on the experimental characterization of 36 *de novo* copper amine oxidases (CuAOs) and 35 de novo retro-aldolases (RADs). Native mass spectrometry revealed that at least three of the CuAOs successfully coordinated a relevant copper ion. Furthermore, native mass spectrometry and protein X-ray crystallography provided evidence supporting the successful formation of a cofactor essential for the reaction. For the designed RADs, previously demonstrated to exhibit high catalytic activity [1], biophysical analyses using circular dichroism spectroscopy and SAXS confirmed their correct α-helical fold and monomeric structure. 34 of the RADs displayed exceptional thermal stability up to 95◦C. Denaturation midpoints were determined to range from 2.5 M to over 6.5 M GdnHCl.

The results of this thesis highlight the immense potential of de novo protein design to create versatile and functional proteins for a wide range of applications, from therapeutics to catalysis. By integrating cutting-edge computational tools with rigorous experimental validation, this work demonstrates the ability to engineer proteins with highly specific and desirable properties, paving the way for future breakthroughs in synthetic biology and biotechnology.

Doctoral Theses completed

Julia Messenlehner: *Computational design of de novo protein pores with custom geometries*

The ability to manufacture de novo proteins for various applications is of great interest and presupposes the understanding of the fundamental principles of the three-dimensional structure of an amino acid sequence. The Rosetta software has been improved over many years and for a decade it was seen as the gold standard regarding the crafting of new backbones and sequence sampling. With its physics-based approach using energy functions and Monte-Carlo sampling a number of proteins were successfully manufactured. Now we are on the edge of the aera of machine learning-based approaches that were trained on experimentally determined protein structures and are already employed to predict structures from sequences and also design fully de novo proteins. In this work a de novo designed soluble homotetramer was designed with Rosetta and later with ProteinMPNN and AlphaFold2 (AF2). In the second chapter Rosetta was used to find positions for the establishment of new disulfide bonds within a given artificial growth factor.

We aimed to design and produce a stable, soluble monomeric, helix bundle (HB) consisting of five helices with a novel fold and design it further to make itself assemble into soluble homo tetramers. In the final design step a membrane spanning pore, once the soluble tetramer was produced and characterized. Since the α-helix, as a secondary structure element, provides highly conserved properties in terms of geometry, and can be fully described by parametric equations it was already shown that highly stable HB built up by three or four helices can be produced successfully. It was shown that the design of membrane proteins with Rosetta is possible. We were able to design and produce a 5HB monomer (Qc762be) comprising a novel helical arrangement and confirmed its structure by CD and SAXS. All efforts to design soluble homo tetramers using the Rosetta software did not lead to a stable protein. In summer 2022, ProteinMPNN [4] was released, and we improved the Rosetta designed sequence towards the backbone of the soluble homo tetramer. We performed three cycles of (re-)design and selected the top hit by comparing the referring AF2 output predictions to the input structure regarding their RMSDs. In between each round, a Rosetta relax run was performed on the predicted structure to diversify the input to some extent.

This strategy finally gave a protein sequence, namely t113, which was predicted with an average pLDDT of 92.4 ± 1.0 and an RMSD to the original input structure of 1.29 ± 0.0.41 over five models. The gene was ordered and the protein with a cleavable His-tag was produced in *E. coli*. Purified with affinity and size exclusion chromatography. All purification steps were carried out in a saline Tris/HCl buffer system, pH 8. Finally, the shape and overall assembly of the tetramer was proofed by SAXS and native MS and a crystal structure with a resolution of 3 Angstroms was obtained (PDB ID: 8R5S). The next steps to yield the membrane protein were tested computationally.

Massimo Totaro: *Bridging physics-based simulations and experimental approaches in bacteriophytochrome protein–protein interface engineering*

This work focused on engineering the dimeric interface of the *Is*PadC bacteriophytochrome photosensory module, using a combination of computational protein design and experimental validation. Computational tools, particularly the Rosetta software suite, were employed to model the protein's structural dynamics and predict how specific mutations would impact its stability and function. Various design strategies were explored and benchmarked to optimize *Is*PadC oligomers light-responsive behavior, enhancing the potential of the protein for optogenetic applications. The engineered constructs were then validated experimentally, demonstrating that targeted modifications at the protein-protein interface effectively modulated photosensory activity in response to light.

The results underscore the remarkable sensitivity of the *Is*PadC system to engineering interventions, emphasizing the importance of leveraging evolutionary information to guide computational design toward successful outcomes. The inherent asymmetry of IsPadC, coupled with the dynamic behavior of its interface, presents promising targets for engineering proteins with enhanced and customizable light-responsive signaling properties. Evolutionarily conserved motifs and structural elements were found to be crucial to preserving the functional integrity of the engineered proteins, reinforcing the necessity of incorporating evolutionary insights into future design strategies aimed at developing effective optogenetic tools.

Moreover, the integration of advanced AI-driven tools offers an up-and-coming frontier in computational protein design, with the promise to significantly enhance the predictive accuracy and efficiency of protein engineering efforts. This work not only highlights the potential of combining computational and experimental approaches but also points to the transformative role that AI could play in the future of protein engineering, particularly in the development of sophisticated optogenetic systems for diverse applications in synthetic biology and beyond.

International cooperations

David Baker, Institute of Protein Design, University of Washington, Seattle, United States

Sinisa Bjelic, Department of Chemistry and Biomedical Sciences, Linnæus University, Småland, Sweden

Anabella Ivancich, Directeur de Recherche CNRS, CNRS et Aix-Marseille Université, France

Anna Peacock, School of Chemistry, University of Birmingham, United Kingdom

Sarel Fleishman, Department of Biomolecular Sciences, Weizmann Institute of Science, Israel

Research projects

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

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

FWF DOC130: "BioMolStruct – Biomolecular Structures and Interactions" doc.funds

Talks at national and international conferences/meetings

1. Oberdorfer, G.: *Ideal is pretty, but irregular more interesting - functionalization of parametrically designed helix bundles by deviating from ideal geometries.* NanoBioPhysiX Club, Wageningen, Netherland, January 2024
2. Oberdorfer, G.: *Machine Learning for Molecules*. GraML Meeting, TU Graz, Graz, Austria, February 2024
3. Oberdorfer, G.: *Ideal is pretty, but irregular more interesting - functionalization of parametrically designed helix bundles by deviating from ideal geometries.* Modern Concepts in Structural Biology Seminar, VIBC, Vienna, Austria, April 2024
4. Oberdorfer, G.: *Ideal is pretty, but irregular more interesting - functionalization of parametrically designed helix bundles by deviating from ideal geometries.* Physical Chemistry Colloquium, LMU Munich, Munich, Germany, May 2024
5. Oberdorfer, G.: *KI-gestützte Lösungen für biotechnologische und biomedizinische Herausforderungen des 21. Jahrhunderts.* Stay Informed Meeting - alumniTUGraz, Graz, Austria, May 2024
6. Oberdorfer, G.: *Modelling and expression of de novo mini proteins.* De novo mini-protein workshop, Marseille, France, July 2024
7. Braun, M.: *Computational design of highly active Retro-Aldolases.* Summer RosettaCon, Suncadia Resort, Cle Elum (WA), USA, August 2024
8. Tripp, A.: *Computational design of highly active Retro-Aldolases.* ÖGMBT Meeting, Graz, Austria, September 2024
9. Wieser, F.: *Assessing the quality of loop structures in predicted protein structures.* ÖGMBT Meeting, Graz, Austria, September 2024
10. Braun, M.: *Computational design of highly active Retro-Aldolases.* Chemietage, Graz, Austria, September 2024
11. Oberdorfer, G.: *Ideal is pretty, but irregular more interesting - functionalization of parametrically designed helix bundles by deviating from ideal geometries*. AminoVerse, online, October 2024
12. Kaltenbrunner, S.: *Computational design of de novo enzymes for the Morita-Baylis-Hillman reaction.* EuropeanRosettaCon, Copenhagen, Denmark, November 2024
13. Oberdorfer, G.: *Practically useful: computational design of highly active de novo biocatalysts.* CuPiD Workshop, Copenhagen, Denmark, November 2024

14. Oberdorfer, G.: *The Nobel Prize for Chemistry 2024 – Computational Protein Design and Protein Structure Prediction.* Colloquium on the Nobel Prizes 2024, Leoben, Austria, December 2024

Publications

1. Totaro, M. G., Vide, U., Zausinger, R., Winkler, A., Oberdorfer, G. *ESM-Scan - a tool to guide amino acid substitutions* (2024) ***Prot. Sci.*** 33, 12, e5221. DOI: 10.1002/pro.5221
2. Tripp, A., Braun, M., Wieser, F., Oberdorfer, G. and Lechner, H. *Click, Compute, Create: A Review of Web-based Tools for Enzyme Engineering* (2024) ***ChemBioChem.*** 25, e202400092. DOI: 10.1002/cbic.202400092
3. Nigl, A., Delsoglio, V., Grgić, M., Malihan-Yap, L., Myrtollari, K., Spasic, J., Winkler, M., Oberdorfer, G., Taden, A., Anić, I. and Kourist, R. *Engineering of Transmembrane Alkane Monooxygenases to Improve a Key Reaction Step in the Synthesis of Polymer Precursor Tulipalin A* (2024) ***bioRxiv*** 2024.07.04.601532; DOI: 10.1101/2024.07.04.601532
4. Braun, M., Tripp, A., Chakatok, M., Kaltenbrunner, S., Totaro, M., Stoll, D., Bijelic, A., Elaily, W., Yakir Hoch, S., Aleotti, M., Hall, M. and Oberdorfer G. *Computational design of highly active de novo enzymes.* (2024) ***bioRxiv*** 2024.08.02.606416; DOI: 10.1101/2024.08.02.606416
5. 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
6. Kastner, K., Bitter, J., Pfeiffer, M., Grininger, C., Oberdorfer, G., Pavkov-Keller, T., Weber, H. and Nidetzky, B. *Enzyme Machinery for Bacterial Glucoside Metabolism through a Conserved Non-hydrolytic Pathway* (2024) ***Angew. Chem. Int. Ed***. 63, e202410681. DOI: 10.1002/anie.202410681

BioTechMed YoungResearcherGroup “Enzyme Design”

Group leader: Horst Lechner
PhD students: Sajith Kolathuparambil
Technical Staff: Ahmed Alagic, Claudia Hrastnik

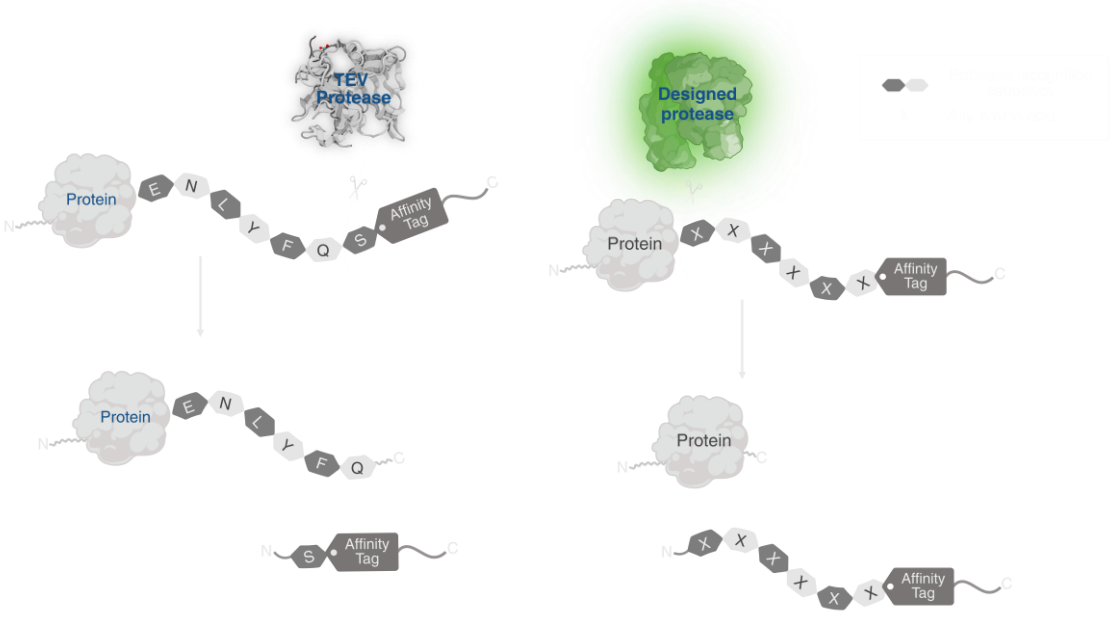
General description

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. A FACS-based screening method is used to select and further improve the *de novo* proteases. (Denard et al., 2021) We established this assay with Tobias Eisenberg (University of Graz). Our computational design pipeline generated dozens of potential proteases. The next steps are to order them as synthetic genes and test them *in vitro*.



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.

Yield enhancement of recombinant enzyme production

Many proteins of interest suffer from marginal stability or limited expression levels in heterologous hosts, posing significant challenges for research and application. This instability not only hinders their use in biocatalysis but also makes them a poor starting point for directed evolution campaigns. Marginal protein stability arises from intrinsic thermodynamic properties and evolutionary pressures specific to the organism's environment. Existing computational tools like PROSS and FireProt2 have demonstrated success in stabilizing proteins and improving yields in recombinant expression. However, these methods rely heavily on multiple sequence alignments, making them less effective for proteins with few homologous sequences or for those originating from organisms that lack selective pressure to evolve stable proteins.

This project aims to evaluate and adapt modern machine-learning-based sequence design algorithms to develop a robust, generalizable method for enhancing recombinant protein production yields and stability. We will utilize advanced tools such as message-passing neural networks (e.g., ProteinMPNN) to improve the production and stability of a model enzyme. This enzyme is difficult to produce in bacterial systems but hold significant potential in biocatalysis.

Ahmed Alagic started as student employee to evaluate computational protein design strategies to improve expression yields of hard-to express proteins. His model enzymes were unspecific peroxygenases.

Research projects

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

TU Graz 20. Anschubfinanzierung: „Diffusion-based design of enzymes“

Talks at national and international conferences/meetings

1. Lechner, H.: *Designed Fluorescent Proteins for Light-Emitting Diodes*, 20. Österreischische Chemietage, Graz, Österreich, September 2024
2. Lechner H.: *Protein Design meets Phosphors for Light-Emitting Diodes*, EMRS Fall Meeting 2024, Warschau, Polen, September 2024

Publications:

1. Tripp, A., Braun, M., Wieser, F., Oberdorfer, G. and Lechner, H. *Click, Compute, Create: A Review of Web-based Tools for Enzyme Engineering* (2024) ***ChemBioChem.*** 25, e202400092. DOI: 10.1002/cbic.202400092