Staff Members of the Institute of Biochemistry, TU Graz

Tel.: +43-(0)316-873-ext.; Mail: office.ibc@tugraz.at

Professors

Peter Macheroux (Full Professor & Head of the Institute)
(peter.macheroux@tugraz.at; Tel.: ext. 6450)

Michael Murkovic (Associate Professor)
(michael.murkovic@tugraz.at; Tel.: ext. 6495)

Gustav Oberdorfer (Assistant Professor)
(andreas.winkler@tugraz.at; Tel.: ext. 6462)

Andreas Winkler (Associate Professor & Deputy Head of the Institute)
(andreas.winkler@tugraz.at; Tel.: ext. 6457)

Senior Research Scientists

Aleksandar Bijelic
(aleksandar.bijelic@tugraz.at; Tel.: ext. 4528)

Silvia Wallner
(silvia.wallner@tugraz.at; Tel.: ext. 6458)

Research Assistants

Stefanie Baldauf
(stefanie.baldauf@tugraz.at; Tel.: ext. 4526)

Katharina Fuchs
(kathrina.fuchs@tugraz.at; Tel.: ext. 6955)

Julia Messenlehner
(julia.messenlehner@tugraz.at; Tel.: ext. 6466)

Ursula Vide
(Ursula.vide@tugraz.at; Tel.: ext. 6467)

Office

Christine Rother
(christine.rother@tugraz.at; Tel.: ext. 6451)

Technical Staff

Macheroux Group:	Eva Maria Frießer (Tel.: ext. 4522), Rosemarie Trenker-El-Toukhy (Tel.: ext. 4522)
Murkovic Group:	Claudia Hrastnik (Tel.: ext. 6996)
Oberdorfer Group:	Birgit Grill (Tel.: ext. 6454) and Alma Makic (Tel.: ext. 6996)
Winkler Group:	Philipp Pelzmann (Tel.: ext. 4528)

Brief History of the Institute of Biochemistry

The *Institute of Biochemistry and Food Chemistry* was born out of the division of the *Institute of Biochemical Technology, Food Chemistry and Microchemistry* of the former *School of Technology Graz*. Together with all the other chemistry institutes, it was located in the old Chemistry Building on Baron Mandell's ground at the corner Technikerstraße-Mandellstraße.

1929 The *Institute of Technical Biochemistry and Microbiology* moved to the Fürstlich-Dietrichstein-Stiftung building, Schlögelgasse 9, the focus of biosciences at that time.

1945 Georg GORBACH - initially in the rank of a docent and soon thereafter as a.o. Professor - took over to lead the new *Institute of Biochemical Technology and Food Chemistry*.

1948 G. GORBACH was nominated full professor and head of the institute. In succession of the famous *Graz School of Microchemistry* founded by PREGL and EMICH, Prof. GORBACH was one of the most prominent and active leaders in the fields of microchemistry, microbiology and nutritional sciences. Questions of water quality and wastewater disposal were the focus of the group of Prof. K. STUNDL. In addition, a division to fight dry-rot supervised by Dr. KUNZE and later by H. SALOMON, was also affiliated with the institute.

1955 In honor of the founder of microchemistry and former professor at *Graz University of Technology*, the labs were called EMICH-Laboratories and the institute was renamed to *Institute of Biochemical Technology, Food Chemistry and Microchemistry*.

1970 After the decease of Prof. GORBACH, Prof. GRUBITSCH was appointed head of the institute. At the end of the 1960s, the division for water and wastewater disposal headed by Prof. STUNDL was established as an independent institute. Prof. SPITZY was nominated professor of general chemistry, micro- and radiochemistry.

1973 Division of the *Institute for Biochemical Technology, Food Technology and Microchemistry* took place. At first, biochemical technology together with food technology formed a new institute now called *Institute of Biotechnology and Food Chemistry* headed by Prof. LAFFERTY.

1973 F. PALTAUF was appointed professor and head of the newly established *Institute of Biochemistry*. The interest of Prof. PALTAUF to study biological membranes and lipids laid the foundation for the future direction of research. G. DAUM, S. D. KOHLWEIN, and A. HERMETTER joined the institute and, after carrying out postdoctoral research in renowned laboratories, established independent research groups in cell biology (G. D.), biophysics (A. H.) and molecular biology (S. D. K.).

1990 The institute moved to a new building in Petersgasse 12. This enabled the expansion of individual research groups, essential for the participation in novel collaborative efforts at the national and international level. Thus, the *Institute of Biochemistry*, together with partner institutes from the Karl-Franzens-University, was the driving force to establish Graz as a center of competence in lipid research.

2003 After Prof. PALTAUF's retirement in 2001, P. MACHEROUX was appointed full professor of biochemistry in September 2003 and head of the *Institute of Biochemistry* in January 2004. His research interests revolve around topics in protein biochemistry and enzymology.

2007 K. ATHENSTAEDT, a long-time associate of Prof. DAUM, received the *venia legendi* for biochemistry. Karin was the first woman to complete the traditional habilitation at the *Institute of Biochemistry*!

2013 Due to faculty reorganizations, the group of J. BOGNER-STRAUSS (formerly at the *Institute of Genomics and Bioinformatics*) joined the *Institute of Biochemistry*. Her main interests revolved around the development and metabolism of adipocytes.

2014 A. WINKLER, who was one of the first students to receive his PhD in the DK "*Molecular Enzymology*", returned to the institute after 5 years postdoctoral research with Prof. Schlichting at the Max-Planck-Institute for Medical Research in Heidelberg.

2015 After more than 40 years at the *Institute of Biochemistry* A. HERMETTER retired in September. As an active scientist he published more than 120 papers in peer-reviewed journals and graduated 25 PhD students.

2016 A. WINKLER was appointed assistant professor at the institute to establish an independent research group in the field of photoreceptor research.

2017 G. DAUM, who had been at the institute for 42 years, retired at the end of the academic year. He was one of the leading scientists in the field of yeast lipid research, enjoying worldwide recognition. He also was an enthusiastic academic teacher supervising 36 PhD students and being director of the *Doctoral School of Biosciences*.

2017 J. BOGNER-STRAUSS left the institute to start a political career first at the federal government and then at the Styrian state government as Councilor for Research, Society, Health and Care.

2019 G. OBERDORFER, who received an ERC Starting grant in 2018, was appointed assistant professor at the Institute of Biochemistry to establish an independent research group in the field of protein design and engineering further strengthening the focus of the institute on protein biochemistry

2021 A. WINKLER received his *venia legendi* in *Structural Biochemistry* and was appointed associate professor at the institute.

2022 H. LECHNER was awarded a BioTechMed Graz Young Researcher Group installation grant. This will allow him to establish his own research group focusing on "*Active site transfer to generate new enzymes*"

Highlights of 2022

In the group of **Peter Macheroux**, the motto was: "Back to the lab!" In September, Peter started a two-months research visit in the group of Prof. Asaph Aharoni (right person in the picture to the left) at the Weizmann Institute of Science in Rehovot, Israel. With the help of Qian Ma (left), Peter (center) learned how to grow *Arabidopsis thaliana* on plant agar plates and in pots. After a few weeks, Qian and Peter successfully generated extracts from leaves and roots of *Arabidopsis* mutants lacking two genes of the berberine bridge enzyme family of monolignol oxidoreductases (AtBBE13/15) with the aim to investigate potential changes in the metabolome. During his stay, he also joined Asaph's group on a day trip to the old town of Jaffa and the rapidly growing city of Tel Aviv.

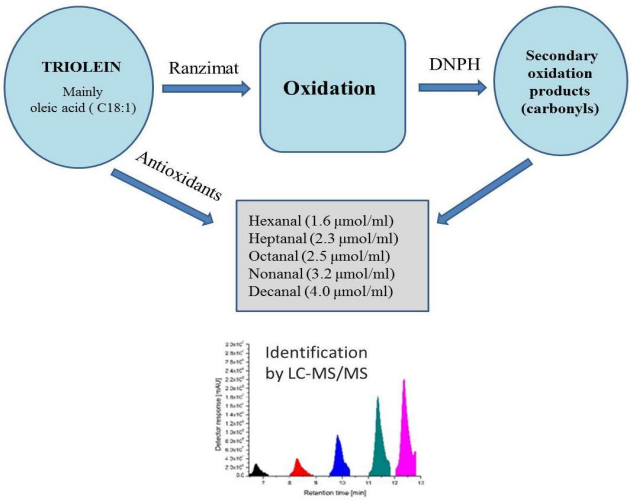
The group of **Gustav Oberdorfer** neither grew, nor shrank in 2022 and still comprises of 14 people. Additions to the group were several bachelor students, including David Stoll, who stayed as crystallography expert ever since. In addition, Melanie Moser joined the group to perform her master thesis on computational design of helical oligomers. Besides that, Horst Lechner, postdoc in the group, received funding from BioTechMed to start his own group.

In the group of **Andreas Winkler,** a return to normal scientific routines was attempted and some group members (Cornelia, Urša, Andreas) went to the *Gordon Research Conference on Photoreceptor Proteins* in Ventura, California. While successful on a scientific level, COVID-19 still left its shadow on this adventure.

In July, some group members also joined the Graz University of Technology dragonboat team "TUG-TUG" and successfully defended the title as winning team in the University cup tournament at the Graz Riverdays 2022. Competing with seven other teams and, after heavy rainfalls, the strong currents of the river Mur, this was a very rewarding adventure.

On a scientific level, several additional seminars and conferences took place and at the end of the year AGW organized its first group retreat. While the weather did not play along and the group enjoyed first snowflakes up in the mountains in early autumn, it was a fruitful team building effort for the group members that had the chance to participate.

In the Functional Food Group of **Michael Murkovic** the main projects were focused on the use of food wastes for cultivation of edible fungi and the development of food ingredients on basis of microalgae. In addition, the cultivation of insects for food is also of interest. In a second project the formation of health threatening components was investigated. These are oxidised lipids and furfuryl alcohol. Both might be related to cancer and a reduction of alimentary exposure should improve food safety. A project on lipophilic alimentary cyclic peptides started.



Biochemistry Group

Group leader: Peter Macheroux
Secretary: Christine Rother
Senior Scientist: Silvia Wallner
Postdoctoral fellow: Aleksandar Bijelic
PhD students: Reinmar Eggers, Katharina Fuchs, Bettina Hierzberger, Bianca Kerschbaumer, Sami Ullah Khan, Grazia Malovan
Master students: Stefanie Baldauf, Anna Lueger, Silvia Kheir
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, nicotineamide) to achieve catalysis. Towards 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 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

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. The model plant *Arabidopsis thaliana*, for example, possesses 27 genes that apparently encode BBE-like enzymes although the plant does not synthesize complex alkaloids. We have recombinantly produced BBE-like homologs, *At*BBE-like proteins 13 and 15, from *A. thaliana* 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 (*AtBBE-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 basis for the design of further experiments. Furthermore, we are working on the generation of 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 *AtBBE-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 *AtBBE-like 24, 25,* and *26*, as these genes are located in close proximity on the same chromosome and the individual mutations can, thus, not be combined in a multiple knockout line via crossing approaches. A 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 obvious that *N. crassa* does possess not only a high number of core flavoproteins participating in housekeeping reactions but also a substantial number of accessory flavoenzymes required for secondary metabolism (Kerschbaumer *et al.*, Front. Catal., 2022). 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 Bianca Kerschbaumer and master thesis project of Silvia Kheir supported by Dr. Silvia Wallner).

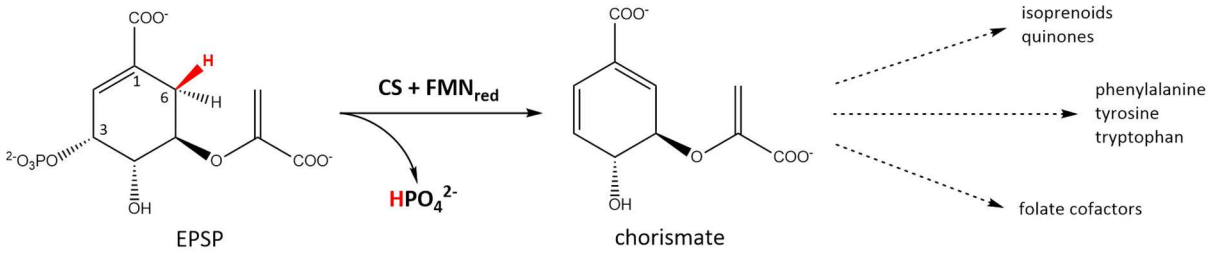
Chorismate synthase as a potential target for pharmaceutically useful inhibitors

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 make this metabolic pathway a promising target for the development of 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 (Rodrigues-Vendramini *et al.*, Antimicrob. Agents Chemother., 2019; Bueno *et al.*, Future Microbiol., 2019). For the design of new inhibitors, the extremely 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 IC50 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 under way.

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 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.

Dipeptidylpeptidase 3

Dipeptidyl peptidase 3 (DPP3) is a zinc-dependent metalloenzyme involved in the catabolism of several bioactive peptides, including the vasoconstrictor peptide Angiotensin II. In view of this, it has been associated with blood pressure regulation and enhancement of cancer cell defense against oxidative stress. The documented efficacy of DPP3 in lowering the blood pressure in angiotensin II-infused hypertensive mice is indicative of its role in cardiovascular diseases, particularly in the protection of hypertension-sensitive organs such as heart and kidney. Recent findings strongly indicate that hemodynamically compromised patients, especially those suffering from myocardial infarction, sepsis, cardiogenic shock and heart failure, display elevated levels of DPP3 in circulation. This increase in circulating DPP3 level

was associated to a higher mortality rate in the patients. Higher circulating DPP3 concentration at admission was also associated with an increased risk of death, circulatory failure, and acute kidney injury in severely burnt patients.

Despite the intriguing involvement of DPP3 in metabolic disease pathogenesis, the underlying physiology of this enzyme is poorly understood (Malovan *et al*., FEBS J., 2022). In collaboration with Prof. Dr. Robert Zimmerman and Prof. Dr. Karl Gruber from the University of Graz, Assoc. **Prof.** Dr. Tobias Madl and Assoc. Prof. Sasa Frank at the Medical University of Graz and Attoquant Diagnostics in Vienna, we uncovered that DPP3 regulates the RAS pathway and water homeostasis by degrading circulating angiotensin peptides. Characterization of a mouse model lacking DPP3 at the genetic and proteomic level showed that this enzyme is associated with heightened water consumption, equilibrium angiotensin peptide levels and renal oxidative stress in mice. Furthermore, we established that DPP3 plays a sex-specific role, where only the male mice presented the phenotype on deletion of DPP3. The female mice were protected from the deleterious effects of DPP3 knockout, indicating a link between the endocrine system and the physiological role of DPP3. The characterization of DPP3 in this study established that it has strong metabolic implications through the modulation of the RAS pathway, a property that could be useful in the management of several cardiovascular and related pathologies. Currently, we are investigating the underlying mechanisms involved in the regulation of these physiological functions by DPP3 using the knockout mouse model and mammalian cell culture. In addition, we are also identifying specific inhibitors of this enzyme through virtual screening and evaluating the obtained hits *in vitro* for their binding affinity to the enzyme as well as their inhibitory properties (Podversnik *et al*., Bioorg. Med. Chem., 2022).

Additional DPP3 *in vitro* experiments were conducted to complement our *in vivo* experiments in collaboration with Dr. Julia Kargl (Medical University of Graz). This includes the use of three different cell lines (MCF-10A, MCF-7 and MDA-MB-231) to explore the cellular processes impacted by DPP3. Currently, we are employing CRISPR/Cas to generate *dpp3* knock-out cell lines to determine the role of DPP3 in viability, migration and apoptosis. In addition, we are interested to check the expression levels of selected proteins and their respective genes that are believed to interact with DPP3, such as Keap1, Nrf2, NQO1 and SOD2. The cell lines are also used to determine the localization of DPP3. (master thesis project of Anna Lueger; PhD project of Bettina Hierzberger and Grazia Malovan).

Oxidative C-H activation for C-C bond formation using the enzymes EasC and EasE

Ergot alkaloids (EAs) are natural products produced by different fungi and widely used in the pharmaceutical industry for drug formulation, and therefore attract attention in various research fields. However, total synthesis of alkaloids is difficult, which is why production of intermediates of the alkaloid synthesis pathway is desired. A crucial step in EA synthesis is a carbon-carbon (C-C) bond formation. In synthetic organic chemistry this step is still challenging as well as time consuming and expensive. Hence, special emphasis has been placed on the field of enzyme catalyzed C-C bond formation in the last two decades.

In order to study the oxidative cyclization in chanoclavine biosynthesis and the involved enzymes and cofactors, we will have a closer look at EasE – a flavin adenine dinucleotide (FAD)-dependent homologue of the berberine bridge enzyme-like (BBE) enzyme – and EasC – a heme-dependent catalase – originating from *Aspergillus japonicus*. CnsA (BBE-like) and CnsD (a putative catalase) will also be investigated, which represent the counterparts of EasE

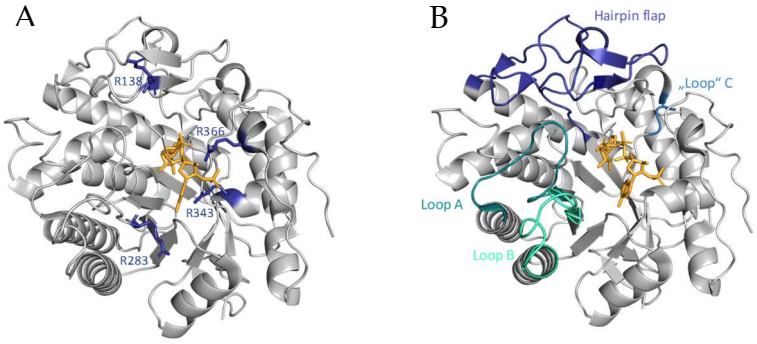
and EasC in *Penicillium expansum*. The primary goal will be the production of the active enzymes by recombinant biotechnology. Furthermore, the obtained proteins will be characterized for their kinetic parameters and their substrate scope. Eventually, the enzymes will be employed to explore their utility in producing derivatives of the fungal alkaloid chanoclavine (thesis project of Bianca Kerschbaumer).

Ene-reductases: shifting the cofactor preference of *Sl*OPR3 from NADPH to 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, the majority of 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).



Crystal structure of *Sl*OPR3 with NADPH (yellow) and labelled mutation target sites (blue, teal): the cofactor is shown as integrated in Dbr2 (PDB: 5dxy), the crystal structure of the closest homolog available with bound NADPH: (A) representation of the mutation sites of the rational design approach, (B) representation of the loop swap approach.

Doctoral Thesis completed

Sami Ullah Khan: *Effects of flavin cofactor modification to 8-formyl-FAD and 8-carboxy-FAD in human electron transferring flavoprotein on electron transfer*

Electron transferring flavoproteins (ETF) are vastly conserved electron transporter, which transmit electrons in mitochondrial fatty acid β-oxidation where they accept one or more electrons from various dehydrogenases and pass them to the electron transport chain. The protein is present immensely in eukaryotic and prokaryotic organisms involving humans (hETF). ETFs are heterodimeric proteins, where extensive protein movement to an open conformation is a prerequisite for effective electron transfer. It is known that lack of the hydrogen bond between αN259 and βE165 results in predominantly open and thus productive form of hETF. Therefore, in-depth biochemical studies on the corresponding αN259A and βE165A variants could help to shed light on electron transfer in this important electron carrier. Moreover, it was reported that the flavin cofactor of ETF can undergo a pH-dependent and irreversible oxidation to its 8-formyl-FAD (8f-FAD) form, which in turn might influence kinetic properties of the enzyme. In my study, I aimed to investigate the effect of FAD cofactor modification and of protein conformation on electron transport in hETF. In course of my studies, I discovered 8-carboxy-FAD (8c-FAD) as a further cofactor modification. It is also reported here that 8f-FAD radical further oxidized to 8-formyl oxidized (8f-FADoxidized) in hETF. Interestingly, the rate of formylation is different between hETF wild type and hETF-αN259A, hETF-ßE156A and hETF-αN259EßE156N variants. I performed activity assays with selected client dehydrogenases, such as the human medium chain acyl-CoA dehydrogenase (hMCAD) and human isobutyryl-CoA dehydrogenase (hiBD) to study electron transfer to the hETF with different cofactor modifications for both hETF wild-type and variants. These kinetic experiments were complemented with theoretical analyses of protein conformation to gain a deeper understanding of this crucial process of electron transfer. These findings could be an indication that the formylation and carboxylation of the FAD cofactor in hETF might play an essential role in modulation of human metabolism since it links various oxidation reactions to respiratory chain in mitochondria.

Master Theses completed

Stefanie Baldauf: *Formation of alamandine using bacterial aspartate-β-decarboxylases (ABDs)*

The Renin-Angiotensin-Aldosterone-System (RAAS) is a hormone system, that is essential for the regulation of blood pressure, fluid homeostasis and electrolyte balance. An important enzyme in this system is ACE2, which converts Ang II to the vasoprotector Ang 1-7. The recently discovered heptapeptide alamandine, a RAAS component, is hypothesized to derive from Ang 1-7, by decarboxylation of the N-terminal aspartate to L-alanine. The enzyme catalyzing this reaction is still unknown, however, the most likely one is a bacterial aspartate-beta-decarboxylase (ABD).

For this thesis four bacterial ABDs and the human aminotransferase were recombinantly expressed in *Escherichia coli* (*E. coli*) to investigate their ability to catalyze the decarboxylation from Ang 1-7 to alamandine. The bacterial enzymes were derived from the organisms *Lactobacillus acidophilus* (*La*), *Pseudomonas* sp. (*Ps*), *Bacteroides thetaiotaomicron* (*Bt*) and *Bacteroides fragilis* (*Bf*), all of which are present in human gut.

The enzymes were purified and characterized with thermal shift assay. The melting temperatures for the bacterial enzymes ranged from 68-76 °C, while the human aminotransferase denatured at 44.5 °C. Kinetic values were determined with a coupled photometric assay for *Ps*ABD and ITC for *Ps*, *Bf* and *Bt*ABD. The Michaelis Menten constants for all enzymes with L-Asp and Ang 1-7 as substrate, ranged from 2.7-23.1 mM. Moreover, also the K_D (0.19-3.08 mM) values for both substrates are in the millimolar range, which implies rather low substrate affinity, resulting in low turnover. From LC-MS results it can be concluded, that the cofactor PLP alone, in an acidic environment yields the highest alamandine production, with 1440 ng/ml. The enzymes *Bf* and *Bt*ABD without additional PLP did not show any detectable conversion of Ang 1-7. *Ps*ABD was tested with 0-100 µM extra PLP, the results ranged from 11-46 ng/ml. The conversion of Ang 1-7 was also analyzed by using an acidic environment for the enzyme, with and without added cofactor. Additional PLP yielded 60 ng/ml alamandine, while the enzyme alone produced 5 ng/ml product. Therefore, free PLP alone can catalyze the conversion significantly better. It is plausible that PLP forms its Schiff base independently from the enzyme and catalyzes the reaction nonenzymatically. The turnover frequencies (TOF) for the LC-MS measurements were in a range of 10^{-5}-10^{-6} per hour. Since the TOF is considerably low, it can be concluded that only a small amount of product is formed in a catalytic cycle.

In conclusion, it was demonstrated with ITC and spectrophotometrically that *Bf*, *Bt* and *Ps*ABD can convert L-Asp and Ang 1-7 to alamandine as well as PLP alone. Since three gut bacterial enzymes could convert Ang 1-7 to alamandine, the hypothesis that the peptide gets decarboxylated in the gut and enters circulation is possible. It is further plausible that PLP in circulation catalyzes the reaction non-enzymatically. However, further in-vivo studies should be performed to obtain a deeper insight in the formation of alamandine in the body.

Anna Lueger: *Dipeptidyl peptidase 3 in MCF-7 and MDA-MB-231 breast cancer cell lines and its role in oxidative stress*

Due to their high metabolic demand, cancer cells are exposed to increased levels of reactive oxygen species (ROS), and therefore, oxidative stress under basal conditions. Dipeptidyl peptidase 3 (DPP3) has an alleviating effect on oxidative stress as a result of its involvement in the Keap1-Nrf2-ARE pathway. In cancer, for instance in breast cancer, high expression of Dpp3 can lead to better survival of cancer cells, and therefore, correlates with poor prognosis. Although DPP3 is a cytoprotective protein that supports the antioxidant system in cells, the presence of DPP3 can have beneficial or harmful effects depending on the pathological condition. The purpose of this thesis was to investigate the importance of DPP3 in breast cancer and to explore the response of breast cancer cells to oxidative stress.

Toward this aim, the breast cancer cell lines MCF-7 and MDA-MB-231 were exposed to hyperoxia and studied through qPCR, western blot, immunofluorescence, cell fractionation, ROS and H_2O_2 measurements as well as several assays, such as the MTT-, TBARS, AnnexinV/PI and the DPP3 activity assay. I could demonstrate that in MDA-MB-231 cells Dpp3 transcript levels can be induced through oxidative stress and that this increase in Dpp3 correlates with survival of cells, as they do not show signs of apoptosis after hyperoxia treatment. This result further supports the hypothesis that DPP3 is cytoprotective and alleviates oxidative stress. Also, I report that the MCF-7 and the MDA-MB-231 cell lines show no cell damage or increase in ROS levels indicating an effective defense of these cell lines against oxidative stress. Furthermore, I show that DPP3 is localized in the nucleus through

immunofluorescence and is independent of oxidative stress. I also detected DPP3 in the nucleus through western blot, however, this should be further analyzed as I could not assess the purity of the nuclear fractions in this experiment. To better understand the localization of DPP3 and its potential as a marker for prognosis in cancer or as a target for cancer therapy, further studies are necessary.

International cooperation

Asaph Aharoni, Weizmann Institute of Science, Rehovot, Israel.

Research projects

FWF P35858: "The family of monolignol oxidoreductases in *Arabidopsis thaliana*"
FWF-doc.funds "CATALOX, Catalytic applications of oxidoreductases"

Publications

1. Saadat, F., Macheroux, P., Alizadeh, H., Razavi, H.: *Economic purification of recombinant uricase by artificial oil bodies* (2022) ***Biores. Bioproc.***, 9, 10, DOI:10.1186/s40643-022-00501-x
2. Macheroux, P.: *More important than ever: understanding how plants cope with stress* (2022) ***FEBS J.***, 289, 1720-1722, DOI:10.1111/febs.16335
3. Malovan, G., Hierzberger, B., Suraci, S., Schaefer, M., Santos, K., Jha, S., Macheroux, P.: *The emerging role of dipeptidyl peptidase 3 in pathophysiology* (2022) ***FEBS J.***, 16429, DOI:10.1111/febs.16429
4. Podversnik, H., Jha, S., Macheroux, P., Breinbauer, R.: *Design and synthesis of efficient fluoroethylene-peptidomimetic inhibitors of dipeptidyl peptidase III (DPP3)* (2022) ***Bioorg. Med. Chem.***, 67, 116831, DOI:10.1016/j.bmc.2022.116831
5. Eggbauer, B., Schrittwieser, J. H., Kerschaumer, B., Macheroux, P., Kroutil, W.: *Regioselective biocatalytic C4-prenylation of unprotected tryptophan derivatives* (2022) ***ChemBioChem***, 23, e202200311, DOI:10.1002/cbic.202200311
6. Skourti, E., Macheroux, P.: *In conversation with Peter Macheroux* (2022) ***FEBS J***, 16646, DOI: 10.1111/febs.16646
7. Kerschbaumer, B., Bijelic, A., Macheroux, P.: *FlavoFun: Exploration of fungal flavoproteomes* (2022) ***Front. Catal.***, 2, 1021691, DOI:10.3389/fctls.2022.1021691

Award

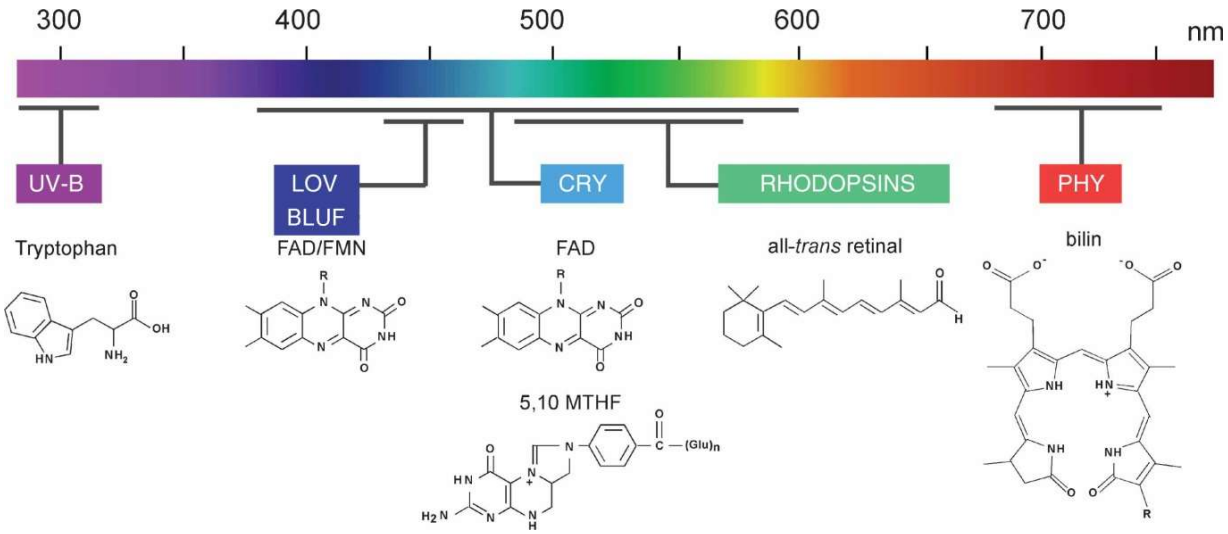
1. Baldauf, S.: Young Chemists Innovation Award – Pitch Your Research, 1st prize in the category Master's students, GÖCH, 2022.

Photoreceptor Group

Group leader: Andreas Winkler
PhD students: Cornelia Böhm, Massimo Totaro (joint with AGO), Hieu Tran, Uršula Vide, Oliver Eder, Maximilian Fuchs
Master students: Martina Reiter (external student at Qualizyme Diagnostics)
Bachelor students: Marta Jančić, Fabienne Mossegger
Project student: Regina Zausinger
Technical staff: Philipp Pelzmann

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.

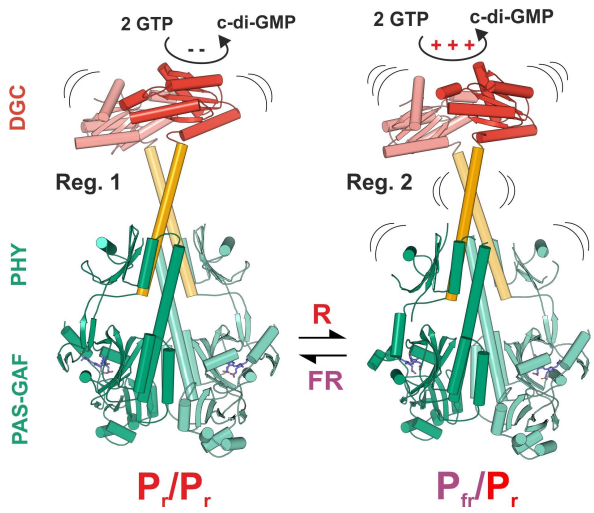


Overview of photoreceptor families and their corresponding cofactors covering the UV/Vis range of the electromagnetic spectrum (adapted from Heintzen WIREs Membrane Transport and Signaling 2012, 1:411–432).

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 the light-regulation of cyclic-dimeric-GMP formation by GGDEF diguanylate cyclases that are ubiquitous in bacteria and 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. Since diguanylate cyclases are distantly related to adenylate/guanylate cyclases, the comparison of signal transduction mechanisms between the two protein families is one central aspect of our research interests.

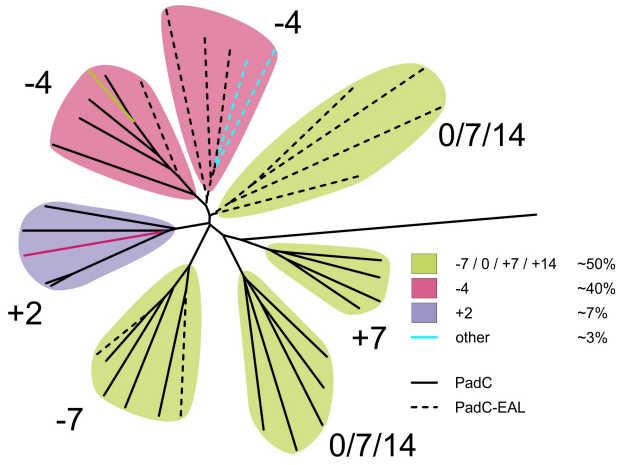
With the elucidation of the first full-length crystal structure of a naturally occurring phytochrome linked to its functional effector domain, new insights into signal transduction in phytochromes were obtained. The structure revealed a parallel dimeric arrangement of the sensor and effector domains. 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 (Gourinchas *et al*., Sci Adv, 2017).



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 (Gourinchas *et al*., eLife, 2018). Understanding the role of asymmetry in these systems is the major focus of the PhD thesis of Hieu Tran.

In an attempt to further characterize molecular determinants of asymmetry in *Is*PadC, Hieu Tran has attempted to use NMR for looking into the conformational dynamics of these complex dimeric systems. Inital 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 fibre-coupled LED via top-down illumination. However, roughened optic fibers and immersion of the fibre into the NMR tube resolved the issue of low activation. However, the propensity of *Is*PadC to transiently form tetrameric assemblies via an “artificial interface” caused severe losses in signal intensity and, thereby, stopped current efforts to follow up on asymmetric properties of light activation via NMR. Other homologs with similar

activation properties, but lacking the artificial interface, might be interesting candidates for follow up experiments with NMR. In an effort to better understand the interplay of specific amino acids with the biliverdin cofactor and to characterize their role in stabilizing Pr or Pfr, Hieu is currently working on an in depth mutagenesis programme of *Is*PadC. Constructs with interesting properties as far as Pr or Pfr stabilities and higher oligomers are concerned might be useful for employing *Is*PadC as an optogenetic tool.



Linker-length clustering in the evolutionary relationship of PadCs (left figure). The phylogenetic characterization carried out by Cornelia Böhm (former PhD student) together with Christoph Sensen, revealed a clustering of linker lengths and a strong conservation of multiples of 7 residues – in line with the coiled-coil character of the sensor-effector linker. The similarly abundant -4 linkers also function as light activated cyclases, albeit with lower dynamic ranges of light activation due to the less optimal coiled-coil architecture. The +2 family features pronouncedly different properties in many aspects, like symmetric Pfr state formation, high light state stabilities and light-inhibition upon illumination.

The central role of asymmetry on PadC function was observed in the biochemical characterization of several homologs from the phylogenetic tree shown in the figure above. While not a strict requirement for PadC functionality, as recently observed in synthetic chimeras (Böhm *et al*. J Mol Biol, 2021), the naturally occurring PadCs with light activation all feature asymmetric red light illuminated spectra. A full characterization of interesting representatives from all major branches of PadCs has been performed by Cornelia Böhm. The biochemical and phylogenetic aspects have recently been published (Böhm *et al*. Photochem Photobiol Sci., 2022) and support the notion that linker length strongly correlates with the functional properties of PadCs.

Optogenetic tools based on red-light sensing phytochromes

Due to deep tissue penetration of red light and the availability of the biliverdin cofactor as heme-degradation product in mammalian cells, bacteriophytochromes are interesting building blocks for optogenetic tools. To assess the suitability of PadC derived systems, we are testing different constructs for their oligomerization characteristics to obtain useful red light regulatable dimerization tools. In addition to the native dimer interface of bacteriophytochromes, artificial interfaces can be employed for improving specificity and bringing together different target functionalities.

In this respect, Massimo Totaro (joint PhD student with AGO) is using Rosetta and deep learning tools (MPNN, ESM, AlphaFold2 etc.) to destabilize the native dimer interface and optimize the artificial interface. Thereby, the complexity of bacteriophytochromes can be reduced and the specificity of the interactions can be increased. Hence the goal of the project is to shift the equilibrium towards a monomeric form in dark conditions and towards an exclusive dimer upon light activation. While destabilizing the native interface interactions proved to be

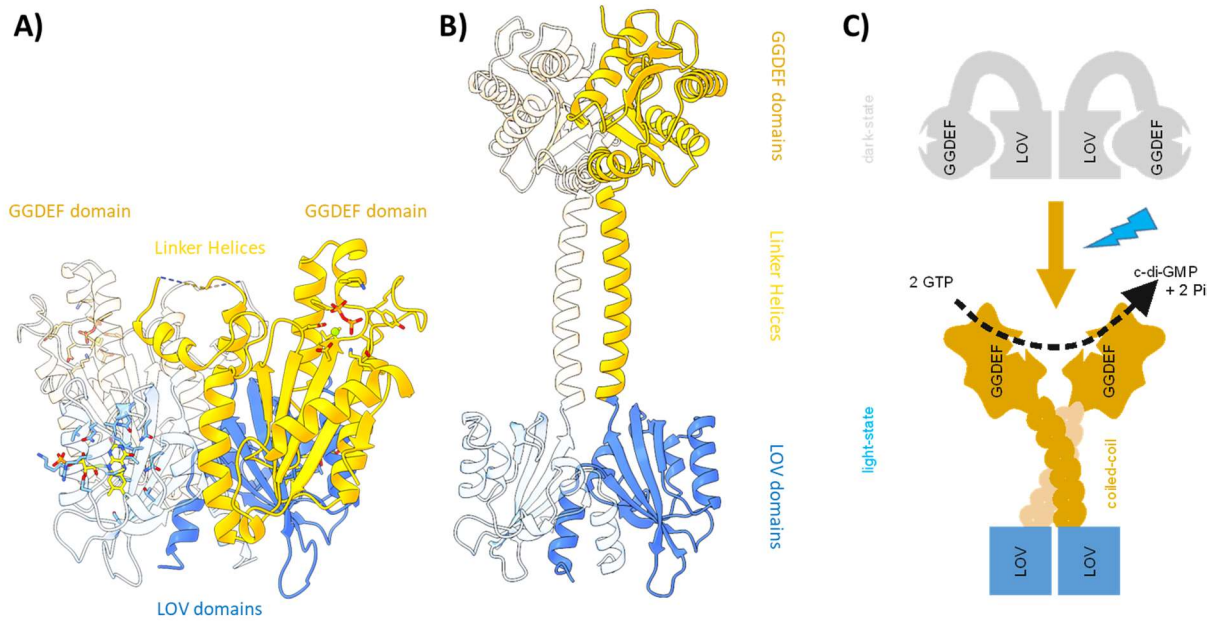
more straightforward, stabilization efforts are potentially hindered by the sensitivity and high dynamic nature of the photo activation cycle. To alleviate such issues more emphasis has been put on evaluating sequence conservation and evolutionary relationships in the design process.

Future efforts also address the possibility to render artificial interfaces specific for heterodimeric interactions. Thereby, one could think of additional applications that are based on the recruitment of different functionalities to induce biological responses. In order to move from the *in silico*-based approach of computational protein design to *in vitro* testing and ultimately *in vivo* applications, Oliver Eder (PhD student), is establishing a screening system in *E. coli* that is based on a bacterial two hybrid system. In parallel, he is testing promising constructs from the calculations that will ultimately serve as controls for the larger scale screening efforts. Various localized sets of amino acid substitutions, concerning dimerization properties have been characterized. In this context, the ability for protein expression, solubility and photo cycling capabilities were the main concerns. Additional efforts are therefore channeled into characterizing close evolutionary homologs and artificial intelligence-designed *Is*PadC PG relatives to reduce these concerns and to get a better feeling for the overall residue context which determines artificial interface interactions.

Blue-light-regulated diguanylate cyclases

Photoreceptors sensing blue light were early on identified as key players in plant phototropism, and belong to the family of flavin-dependent light-oxygen-voltage (LOV) domains (Losi & Gärtner, Photochem. Photobiol. Sci, 2017). LOV domains have a high potential in applied biosciences, as they contributed significantly to the field of optogenetics using a variety of LOV-coupled effector domains (Pudasaini *et al*., Front. Mol. Biosci. 2015).

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 (Vide *et al*., manuscript submitted). Additional aims of the PhD thesis of Uršula Vide go 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 at the sensor-effector linker and at the extensive inhibitory interface between the two domains, she is currently also studying computer-designed LOV domains in a collaboration with Massimo G. Totaro.



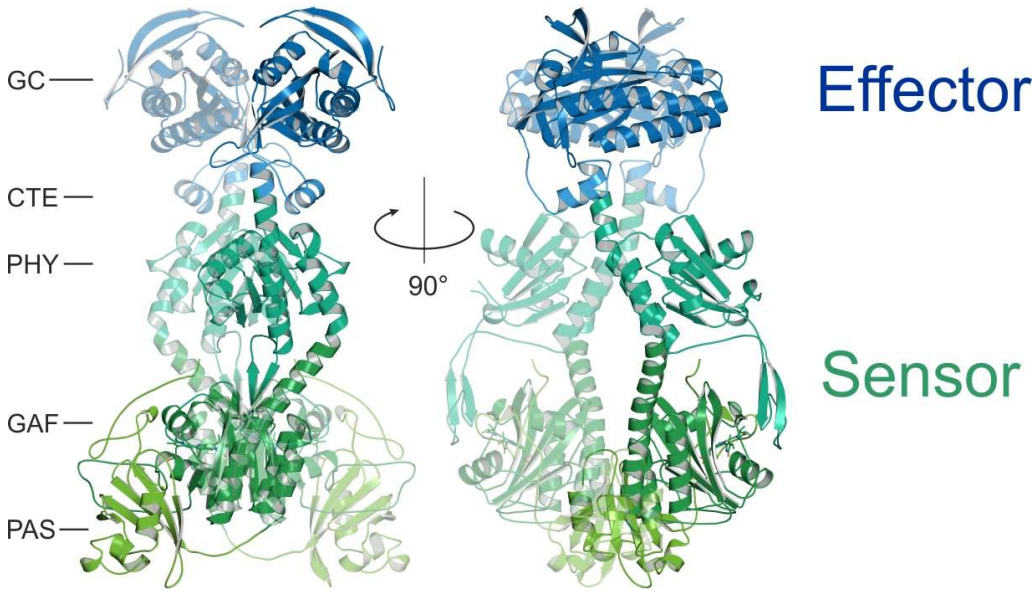
Basis for the LOV-activated diguanylate cyclase switch mechanism. Crystal structure (**A**) of a LOV-GGDEF homolog and its predicted AlphaFold model (**B**) showing a coiled-coil sensor-effector linker. The dark, inhibited conformation (**A**) is characterized by a tight association of the sensory LOV 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**).

Furthermore, diguanylate cyclases can be regulated by a variety of other stimuli. More complex LOV-GGDEF-EAL or Rec-LOV-GGDEF systems are interesting to address molecular logics of sensor-effector systems with multiple inputs or output functionalities (PhD project of Maximilian Fuchs). Fundamental questions of how these multi-domain proteins are linked will be analyzed by looking at phylogenetic aspects, which will help to find clusters of proteins with similar characteristics based on their sequences. Currently, Rec-LOV-GGDEF systems are being studied and are referred to as dual-sensor diguanylate cyclases. For the Rec domain, in contrast to our light-regulated input domain (LOV), phosphorylation will lead to either active or inactive conformations. Only a few dual-sensor systems have yet been characterized. Therefore, a focus is set on unraveling the molecular mechanism of signal processing in these proteins. X-ray crystallography is currently used for structure elucidation and has led to promising preliminary data from a Rec-LOV truncation construct. Understanding the rules, which dictate cooperative domain interplay, would help to create molecular tools that can implement molecular logics for adding additional regulatory networks in optogenetics.

Light-activated adenylate/guanylate cyclases

Another area of research are red-light activatable guanylate and adenylate cyclases, respectively. These represent artificial light-regulatable tools that are designed based on functional data of a blue-light regulatable adenylate cyclase (Lindner *et al*., J Mol Biol, 2017) and the evolutionary conservation of elements required for signal transduction. The identification of specific signaling elements for the closely related effector systems and the comparison of functionalities of different artificial chimeras provided new insight into the coupling mechanism of sensor-effector modules. The successful characterization of these

systems required an interdisciplinary approach combining biochemistry with tools of structural biology. Atomic models obtained from x-ray crystallography were functionally extended by the in-solution method hydrogen-deuterium exchange coupled to mass spectrometry (HDX-MS) to obtain structural information of elements that are involved in photo-activation and signal transmission. Highlights in this project were the elucidation of a full-length structure of a functional red-light activated adenylate/guanylate cyclase (see figure below) and the demonstration that it can be used as an effective optogenetic tool in the animal model system *Caenorhabditis elegans* (Etzl *et al*., J Biol Chem, 2018).



Crystal structure of the bacteriophytochrome-linked adenylate/guanylate cyclase. PAS, GAF and PHY domains are part of the red light regulated phytochrome sensor with its biliverdin chromophore shown as green stick model. The CTE corresponds to the Cyclase Transducer Element, required for efficient signal integration by the adenylate/guanylate cyclase (GC) effector.

In addition to these efforts, specific sensor variants were created that are supposed to result in functionally uncoupled enzymatic activities (Takala *et al*., J Biol Chem 2018). In order to further address the role of the sensor-effector linker, different linker length constructs were analyzed in detail. While showing a high dynamic range of activation in the wild-type form, the uncoupled variants displayed either complete loss of enzymatic activity or a relatively high basal activity. To this end we teamed up with Janne Ihalainen at the University of Jyväskylä (Finland) and could show that the composition of the effector domain influences the stability of the hairpin extension. The hairpin, often also referred to as the PHY tongue, is one of the central structural elements for signal transduction. It extends from the PHY domain and establishes close contacts with the chromophore binding site in the GAF domain. If the coupling between these interactions is disrupted, the dynamic range of the enzymatic regulation is reduced. Our study highlights the complex conformational properties of the hairpin extension as a bidirectional link between the chromophore-binding site and the output module, as well as the functional properties of diverse output modules linked to the same sensory module (Kurttila et al., Photochem. Photobiol. Sci., 2022).

Methodological developments and 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 (light regulation – see above), biomolecular interactions (together with Monika Oberer, UG), or immobilization on carriers (collaboration with Bernd Nidetzky).

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.

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 modifications can be readily identified. Considering the famous 4th commandment of enzymology „Thou shall 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.

PhD thesis completed

Cornelia Böhm: *Characterisation of Sequence-Structure-Function Relationships in Phytochrome-Regulated Diguanylate Cyclases*

Bacteriophytochromes are a diverse group of photoreceptors, characterized by their sensitivity to red and far-red light. A multitude of effectors is found naturally coupled to phytochrome sensors, highlighting the modularity and malleability of the photosensory core. Integration of a light stimulus and subsequent regulation of output functionality is based on various signal transduction pathways. In the family of phytochrome-activated diguanylate cyclases (PadCs), productive encounter of the GGDEF effector domains defines generation of the bacterial second messenger cyclic dimeric GMP. Even within this highly specific subgroup of bacteriophytochromes, however, a remarkable heterogeneity of both structural and functional characteristics is observed. In light of such diversity, detailed characterisation of input and output features on a molecular level becomes a prerequisite for truly understanding PadC photoactivation. I address this challenge with an integrative approach. Comprehensive biochemical evaluation of chimaeras of two fundamentally different PadC homologs is complimented by a more global analysis of sequence-structure-function relationships.

Hydrogen-deuterium exchange further enables the analysis of conformational dynamics in these photoreceptor proteins. The compiled results highlight the PHY-specific domain dimer interface in tight interplay with the coiled-coil linker element as crucial players in red light-governed modulation of enzymatic activity. The PHY domain features low sequence conservation and serves as a malleable functional integrator unit. In contrast, the length of the linker element is under considerable evolutionary pressure to preserve or terminate coiled-coil register switching as a signal processing mechanism. Regulation of enzymatic turnover is further defined by global conformational dynamics and a complex interplay of several key elements. While molecular details of cofactor activation and conformational changes in its surroundings are in line with what has been described for other bacteriophytochromes, functional signal integration in PadCs does not correspond to any other mechanism observed for phytochrome-coupled photoreceptors. The additional insights gained on PadC signalling further highlight the complexity of bacteriophytochrome photoactivation, and detailed characterisation of the mechanisms at work remains a challenge. Using integrative approaches of sequence, structure and function analyses, I provide a deeper understanding of biliverdin-regulated modulation of diguanylate cyclase activity and thus a valuable contribution to appreciation of sensor-effector modularity.

Master thesis completed

Martina Reiter: *Biochemical characterisation of a chromogenic peptidoglycan-based substrate for lysozyme*

In the shadow of the current challenges regarding the coronavirus, another pandemic emerges as a serious problem for human health: bacterial antimicrobial resistance (AMR). Now already, infections with antibiotic-resistant bacteria are the third most frequent cause of death worldwide. By the year 2050, even 10 million deaths are estimated to be attributable to AMR. To combat this severe issue, a wide range of countermeasures is implemented. Among others, one strategy is the acceleration of the research on rapid diagnostics. In one of these diagnostic tools being under development, a chromogenic, peptidoglycan-based substrate is used to detect lysozyme, which is a marker for infections produced naturally in the body. However, the exact nature of the interaction between the reactive dye Remazol Brilliant Blue R which is used for labelling and peptidoglycan from *Micrococcus luteus* and the effect of the labelling on lysozyme cleavage behaviour remain unknown. The results of this thesis show a broad variety of digestion products identified via reversed-phase HPLC coupled with MS and UV/Vis analysis, depending on various parameters like incubation time with lysozyme and staining degree of the substrate. Furthermore, a covalent linkage between the label and peptidoglycan, more specifically the amino group of the lysine side chains of peptidoglycan pentapeptides, could be verified. The findings of this work contribute to a better understanding of the substrate and thereby of the diagnostic test. In the future, the use of this tool might allow wound and joint infections to be detected more quickly and reliably, hence preventing the misuse of antimicrobial agents.

This master's thesis was carried out in collaboration with Qualizyme Diagnostics, Graz, Austria.

International cooperations

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

Janne Ihalainen, University of Jyväskylä, Jyväskylä, Finland

Han Remaut, VIB-VUB Center for Structural Biology, Belgium

Vladislav Verkhusha, Albert Einstein College of Medicine, New York, USA

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"

Talks at national and international conferences/meetings

1. Vide, U.: *Naturally occurring ON-OFF switches: Blue light-regulated LOV-diguanylate cyclases*. 26th Doc-Day of the Doctoral School of Molecular Biomedical Sciences and Biotechnology, Graz, Austria, February 2022.
2. Böhm, C.: *Characterization of Sequence-Structure-Function Relationships in Phytochrome Regulated Diguanylate Cyclases*. Gordon Research Seminar on Photosensory Receptors and Signal Transduction, Ventura, California, U.S.A., March 2022.
3. Winkler, A.: *Unraveling molecular details of sensor-effector communication in bacteriophytochromes via integrative approaches*. Gordon Research Conference on Photosensory Receptors and Signal Transduction, Ventura, California, U.S.A., March 2022.
4. Eder, O.: *Developing novel red light sensitive protein dimerization tools*. 1st Scientific Retreat - BioMolStruct, Seggau, Austria, June 2022.
5. Fuchs, M.: *Expression and characterization of dual sensor diguanylate cyclases*. 1st Scientific Retreat - BioMolStruct, Seggau, Austria, June 2022.
6. Tran, Q.H..: *Analysis of conformational dynamics of red/far-red light activated bacteriophytochromes*. 1st Scientific Retreat - BioMolStruct, Seggau, Austria, June 2022.
7. Böhm, C.: *Characterization of Sequence-Structure-Function Relationships in Phytochrome Regulated Diguanylate Cyclases*. 27th Doc-Day of the Doctoral School of Molecular Biomedical Sciences and Biotechnology, Graz, Austria, July 2022.

Publications

1. Böhm, C., Gourinchas, G., Zweytick, S., Hujdur, E., Reiter, M., Trstenjak, S., Sensen, C.W., Winkler, A..: *Characterisation of sequence–structure–function space in sensor–effector integrators of phytochrome-regulated diguanylate cyclases* (2022) ***Photochem. Photobiol. Sci.*** 21 (10) 1761-79. DOI: 10.1007/s43630-022-00255-7.

2. Kurttila, M., Etzl, S., Rumfeldt, J., Takala, H., Galler, N., Winkler, A., Ihalainen, J.A.: *The structural effect between the output module and chromophore-binding domain is a two-way street via the hairpin extension* (2022) ***Photochem. Photobiol. Sci.*** 21 (10) 1881-1894. DOI: 10.1007/s43630-022-00255-7

3. Vide, U., Kasapović, D., Fuchs, M., Heimböck, M.P., Totaro, M.G., Zenzmaier, E., Winkler, A.: *Illuminating the inner workings of a natural protein switch: Blue-light sensing in LOV-activated diguanylate cyclases* (2022) ***under consideration***

Awards

1. Vide, U.: *Best Presentation Award*. 26th Doc-Day of the Doctoral School of Molecular Biomedical Sciences and Biotechnology

2. Böhm, C. *Best Presentation Award*. 27th Doc-Day of the Doctoral School of Molecular Biomedical Sciences and Biotechnology

Protein Design Group

Group leader: Gustav Oberdorfer
Postdoctoral fellow: Horst Lechner
PhD students: Veronica Delsoglio, Wael Elaily, Julia Messenlehner, Florian Wieser, Massimo Totaro, Adrian Tripp, Markus Braun
Master students: Nina Grujicic (finished September 2022), Henrik Seyfried, Melanie Moser
Bachelor students: David Stoll, Oliver Damm, Theresa Gogg, Kerstin Zeber, Ahmed Alagic, Barbara Millonig
Technical Staff: Alma Makic, David Stoll, Katharina Leitner, Birgit Grill

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.

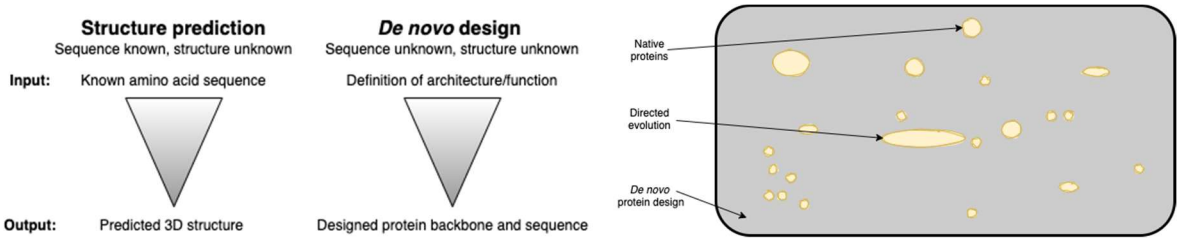


Figure (left) *Differences in structure prediction and de novo protein design* ***(right)*** *Illustrative representation of protein sequence space (grey). Sequence analysis of native proteins shows that tight clusters of protein families can be identified (beige).*

Highlight: Artificial fluorescent proteins for the next generation of sustainable and safer LEDs (ARTIBLED)

The European Union (EU) priority action lines state the need of efficient electricity consumption, expecting >20% reduced consumption using inorganic white light-emitting diodes (WLEDs) present in the LED lamps of our homes [1]. They consist of a blue-chip covered by color down-converting filters based on toxic and/or rare-earth elements (inorganic phosphors or IPs) that are, indeed, listed as one of the 27 critical raw materials by the EU. It is expected that reserves of IPs will run out in 10-15 years if global demand for LEDs rises as expected, while the recycling process is still quite inefficient. The rising costs and ecological impact (mining/refining/toxicity) along with the control of the limited rare-earth resources put a high burden on the long-term sustainability of WLEDs [2]. Finally, the IP filters do not efficiently convert blue light, causing serious implications on visual acuity in children and elderly people and non-visual effects, such as sleep disorder in adults. We must be aware that we are using artificial illumination for long periods of time throughout the day (8-14 h per day).

In this context, EU/US Governments command to replace IPs by non-toxic and sustainable organic phosphors (OPs) towards a new generation of hybrid WLEDs. Since their first introduction in 1995, polymers, carbon nanodots, and fluorescent dyes embedded into polymer matrices have been applied as OPs without meeting customer requirements, showing, in general, low efficiencies and stabilities. This is related to i) the reduction of the photoluminescence quantum yield (PLQY) due to aggregation-assisted quenching and ii) the degradation upon both UV/thermal curing of the matrix and photo-induced oxidation under ambient operation conditions.

A joint research FET-OPEN initiative (ARTIBLED) between Spain (CIC-biomaGUNE-Dr. Cortajarena; CFM-CSIC-Dr. Coto), Italy (UNITO-Dr. Barolo; ABIEL-Dr. Ghersi), Austria (TU Graz-Dr. Oberdorfer), and Germany (TUM-Dr. Costa) aims at replacing IPs by protein filters based on engineered fluorescent proteins (FPs) for lighting applications. The first milestone was realized by the research team of Dr. Costa (TUM) that introduced a new concept to stabilize FPs into different polymer matrices for long periods of time under ambient storage and operating conditions in WLEDs [3]. This new technology is called Bio-LED and has already achieved up to 6 months of stability with a high efficiency [4]. In order to further improve this technology, ARTIBLED promises to achieve a second milestone focused on developing a new family of artificial fluorescent proteins customized for our lighting needs.

The key element for this vision is a true cross-disciplinary work. Within ARTIBLED we are combining expertise in organic synthesis of fluorescent dyes, quantum chemistry calculations, protein design and synthetic biology, material sciences as well as process

development. To achieve our goal, we first simulate thousands of different hypothetical protein scaffolds that are designed to bind specifically to new synthetic dyes without affecting their photophysical properties. A subset of all designed and *in silico* evaluated structures are then ordered as synthetic DNA constructs and experimentally tested for dye binding and photophysical properties. As soon as lead candidates are identified, these new, artificial fluorescent proteins are integrated into a polymer matrix and tested for their suitability as color down-converting filters with regards to Bio-LEDs (Figure below) This all is made possible through recent research and technology advances in quantum chemistry calculations, protein design and synthetic biology, the synthesis of fluorescent dyes, and the invention of novel polymer coating for Bio-LEDs.

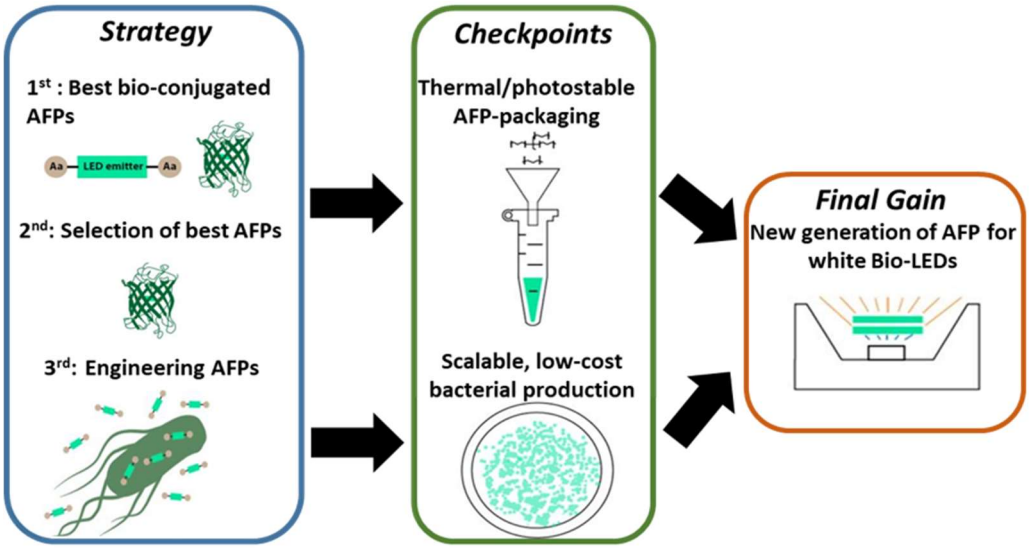


Figure. *The ARTIBLED concept – We start by engineered artificial amino acids into selected protein scaffolds for covalent labelling with the fluorescent dyes. This is followed by experimental testing of this host-guest system for its fluorescent properties. Next, the labelled protein is packaged into a polymer matrix, which is subsequently put in front of the LED chip as down-converting filter.*

Fluorescent dyes and Quantum Chemistry

A key aspect in the development of new biomaterials for lighting applications is the design and synthesis of optimized dyes featuring excellent photoluminescence and photostability. Well-designed emitters, when incorporated in the protein environment, should keep or even enhance their optical features, attaining higher stability. Therefore, the synthetic chemist group (UNITO-Dr. Barolo) plays a key role in the development of compact biocompatible emitters of proven photostability. The second important step is to develop an easy scale-up and low cost synthetic pathway within a greener process. In this respect, theory and simulation (CFM-CSIC-Dr. Coto; TU Graz-Dr. Oberdorfer) play an important role in the development of new dyes for their use in protein-based lighting systems. On the one hand, they can provide a link between molecular and electronic structure of the dye, its stability and optical properties, and how these are modified by the surrounding environment, thus facilitating the design and synthesis of dyes exhibiting specific characteristics. On the other hand, they can provide detailed information on the processes that occur during device operation, assisting the interpretation of experimental results and providing the underlying mechanistic information that cannot be straightforwardly obtained from experiments. (Figure below).

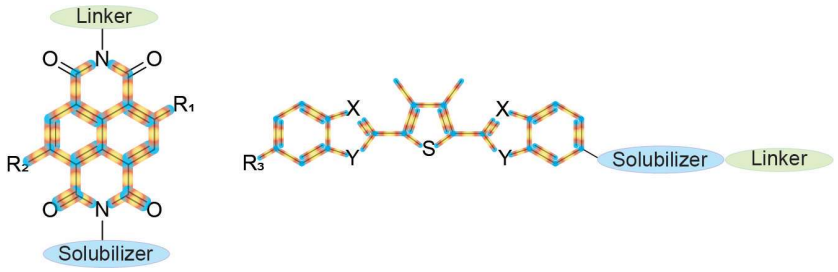


Figure. *Backbones of the synthesized dyes with fine-tuned photophysical features, able to specifically tag protein scaffolds*

Protein Design and Synthetic Biology

In the last decade, the fields of protein design and synthetic biology are driving significant advances towards robust engineering of novel synthetic biomolecular systems [5]. These approaches have been applied mostly to the fabrication of synthetic systems for biomedical applications and less effort has been put on using this technology for the biotech sector. However, the state-of-the-art in those fields suggests their readiness to face relevant technological challenges. In this sense, computational protein design presently enables the generation of stable *ad hoc* protein scaffolds both *de novo* as well as based on existing protein templates. Especially *de novo* proteins have proven to be of very high thermal stability (melting temperatures above 100°C) [6]; a property often highly desired for biotechnological applications. Similarly, synthetic biology tools, including the unnatural amino acids (UAA) technology, in which an orthogonal tRNA-Synthetase system is used to introduce a novel/unnatural amino acid into a protein synthesized by the ribosome, permit the incorporation of novel chemical functionalities into proteins, thereby expanding the molecular toolbox for potential synthetic systems enormously. Altogether, the biomolecular engineering groups (CIC-biomaGUNE-Dr. Cortajarena; TU Graz-Dr. Oberdorfer) are well equipped to tackle the current challenges faced in the technological application of protein-based components for lighting applications (Figure directly below).

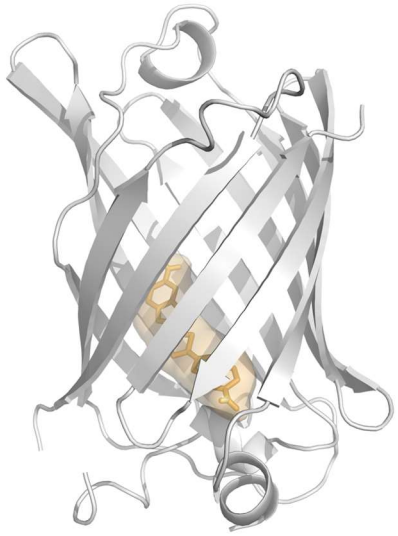


Figure. *Rendering of a fluorescent dye bound to a designed beta-barrel protein. The fluorescent dyes are covalently attached to the protein via the introduction of an artificial amino acid residue. During the design process all surrounding residues will be optimized computationally to generate an ideal environment for the dye.*

Environmentally friendly polymer coatings for lighting purposes

The interest in using protein scaffolds stems from i) their ability to provide an excellent shield of the chromophore to the ambient surrounding, and ii) an essentially limitless bacterial production in-place and highly optimized. However, the main bottleneck is the low stability of protein scaffolds out of the cellular environment and/or physiological conditions in aqueous solutions. This has been solved by a material scientist group (TUM; Dr. Costa) using an innovative stabilization method combining branched/linear polyethylene derivatives to replace the water molecules surrounding the protein skeleton using physical cross-linking upon drying. In this way, the protein scaffold holds its bio-functionality in an easy-to-manipulate polymer material to further fabricate filters for lighting applications. First Bio-WLEDs consisted of a blue-LED chip covered by several natural FPs like enhanced green fluorescent protein and mCherry, which partially converts blue into green and red, achieving efficiencies >50 lm/W, a color rendering index (CRI) >80, and stabilities >100 h (Figure below) [3]. Further optimization (architecture/polymers/proteins) led to >3000 h at >130 lm/W [4]. Thus, solutions to pH, temperature, and oxidative driven degradations are at hand. However, the chromophores present in natural FPs show a prominent photodeactivation (H-transfer/*cis-trans* isomerization) in the polymer matrix. Thus, the redesign of the protein with respect to its chromophore could be key towards highly stable and efficient bio-based lighting systems.

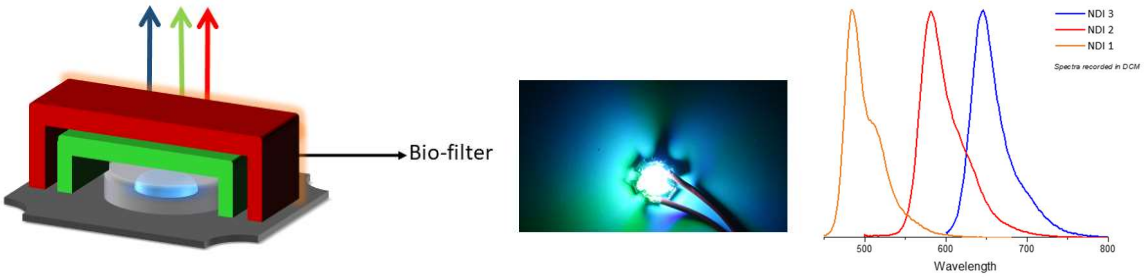


Figure. (left) *Schematic drawing of Bio-WLED and picture of a running prototype.* ***(right)*** *Emission spectra of the dyes currently used.*

Large scale production

To test our artificial fluorescent proteins for larger scale production, the industrial partner (ABIEL-Dr. Ghersi) will test the productive transfer of the new fluorescent proteins from lab scale batch production in shaker flasks to a fermenter based scale-up in order to determine their pre-industrial production in terms of quantity and quality. It is the goal of the project to come up with a production and purification processes with highly competitive costs compared to current standards. ABIEL has acquired experience in the field of macromolecule synthesis that has led to the patenting of this procedure for some enzymes of which it is a producer [7], representing a high yield production and purification procedure with low costs.

Conclusion

The invention of WLEDs is considered the future of artificial lighting, as we are now replacing all the old-fashioned lamps/bulbs by the highly efficient and stable LED technology. However, this comes at high ecological cost by utilizing scarce materials that are not sustainable in the long-term. Within ARTIBLED we have compiled a cross-disciplinary consortium to

develop the next generation of color filters with the aim to boost the emerging Bio-LED technology. Based on our innovative approach, our goal is to keep the actual device performance combining artificial fluorescent proteins and polymers that are environmentally friendly, low cost, and sustainable. Thus, the ARTIBLED consortium expects to realize a significant thrust to all the above subfields, while achieving a well-oriented technological goal: cheap/in-place production of bio-materials for lighting. This is a unique approach that, if successful, will shape the future of our home illumination.

References

[1] a) Eurostat, June **2017**; b) EU Commission, Dec. **2017**.

[2] a) CADMUS, *http://ma-eeac.org/wordpress/wp-content/uploads/MA-Task-5b-LED-Incremental-Cost-Study_FINAL_01FEB2016.pdf*, Feb. **2016**; b) US Department of Energy https://energy.gov/sites/prod/files/2017/09/f37/ssl_suggested-research-topics_sep2017.pdf; Sep. **2017**.

[3] a) M. D. Weber, *et al. Adv. Mater.*, **2015**, *27*, 5493; b) L. Niklaus, *et al. Adv. Funct. Mater.* **2017**, *27*, 1601792; c) C. Fernández *et al. ACS Omega*, **2018**, *3*, 15829; d) V. Fernández-Luna, *et al. Angew. Chem., Int. Ed.* **2018**, 57, 8826.

[4] *A. Espasa et al. Nat. Commun.* **2020***, 11, 879*

[5] a) P. Huang, *et al. Nature* **2016**, 537, 320–327 b) Chin, J. *et al. Nature* **2017**, 550, 53–60

[6] P-S. Huang*, G. Oberdorfer*, *et al. Science* **2014***,* 346, 481-485. *equal contribution

[7] *Bertuzzi, F., Cuttitta, A., Ghersi, G., Mazzola S., Salamone M., Seidita G., PCT WO 2011/073925 USA, Europe patent no. EP10818077.9, Canada patent n. 2784050 and Israel patent no. 220359)*

Young Research Group

Horst Lechner: *Active site transfer to generate new enzymes*

For his outstanding proposal to design new, specific proteases by equipping peptide binding proteins with active sites from proteases, Horst Lechner was awarded a BioTechMed Graz Young Researcher Group grant. He will use this grant to establish his own research group at the department – a great addition of complementary research for the department.

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

Research projects

FWF P30826: “Design of de novo protein pores with custom geometries”

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

ERC FET-OPEN (863170), Participant: “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. Designed Metalloproteins. April 2022, Marsaille, France
2. Oberdorfer, G.: *Ideal is pretty, but irregular more interesting* - functionalization of parametrically designed helix bundles by deviating from ideal geometries. Alpbach Meeting on Coiled Coils, Fibrous and Repeat Proteins. September 2022, Alpbach, Austria
3. Oberdorfer, G.: *Ideal is pretty, but irregular more interesting* - functionalization of parametrically designed helix bundles by deviating from ideal geometries. CuPiD Workshop, September 2022, Alpbach, Austria
4. Oberdorfer, G.: *Ideal is pretty, but irregular more interesting* - functionalization of parametrically designed helix bundles by deviating from ideal geometries. ESIB (European Summit of Industrial Biotechnology) November 2022, Graz, Austria

Publications

1. Pfeiffer, M., Crean, Rory M., Moreira, C., Parracino, A., Oberdorfer, G., Brecker, L., Hammerschmidt, F., Kamerlin, S. C. L. and Nidetzky, B. Essential Functional Interplay of the Catalytic Groups in Acid Phosphatase. (2022) ***ACS Catalysis***, 12 (6), 3357, DOI: 10.1021/acscatal.1c05656
2. Daniel, B., Hashem, C., Leithold, M., Sagmeister, T., Tripp, A., Stolterfoht-Stock, H., Messenlehner, J., Keegan, R., Winkler, C. K., Guyang Ling, J., HH Younes, S., Oberdorfer, G., Bakar, F. D. A., Gruber, K., Pavkov-Keller, T. and Winkler, M. *Structure of the Reductase Domain of a Fungal Carboxylic Acid Reductase and Its Substrate Scope in Thioester and Aldehyde Reduction*. (2022) ***ACS Catalysis*** 12 (24), 15668, DOI: 10.1021/acscatal.2c04426
3. Lechner, H., Oberdorfer, G. *Derivatives of natural organocatalytic cofactors and artificial organocatalytic cofactors as catalysts in enzymes*. (2022) ***ChemBioChem*** 23 (13), e202100599, DOI: 10.1002/cbic.202100599

Chemistry of Functional Foods

Group leader: Michael Murkovic
PhD students: Monika Grasser
Master students: Iris Stalzer, Zehra Gromilic, Monika Kozlowska, Adnan Fojnica, Vildana Fazlic, Zerina Duhovic, Franz Lindbichler
Technical staff: Claudia Hrastnik, Lena Pfeiffer

General description

Antioxidants have different functions depending on the location of action. Is it the protection of biological systems maintaining the integrity of the system or the protection of foods against oxidation leading to health threatening substances? The exposure to oxidation products is either described as oxidative stress or the oxidized substances have an acute or chronic toxicity or are carcinogenic. The production of healthier and safer foods is of primary interest of this research group.

The antioxidants of interest are polyphenols including anthocyanins and carotenoids. The evaluation of their occurrence in food and their behavior during processing and cooking is important especially when these substances are used as food additives. The safety evaluation of these compounds includes the evaluation of possible degradation products.

Heating of food is a process that is normally done to improve the safety and digestibility and improve the sensory attributes like texture, color, and aroma. During the heating reactions occur that lead to the degradation of nutritive constituents like carbohydrates, proteins, amino acids and lipids. Some of the reaction products are contributing to the nice aroma, color, and texture of the prepared food and many of them are highly toxic and/or carcinogenic. However, these hazardous compounds occur in rather low concentrations being normally not acute toxic. The substances have a very diverse chemical background like heterocyclic amines, polycondensated aromatic compounds, acrylamide, or furan derivatives. The aim of the research is to investigate the reaction mechanisms that lead to the formation of these hazardous compounds and establish strategies to mitigate the formation and thereby reducing the alimentary exposure. Recent results show that oxidized lipids are related to either colon cancer or hepatic inflammation. With this background a project was started to investigate the formation of non-volatile aldehydes during lipid oxidation in edible oils and during roasting of coffee.

A new research area was started in which the presence of cyclic lipophilic alimentary peptides is investigated. These cyclic peptides can have specific physiological properties with a positive effect on e.g. malaria.

Master Theses completed

Farah Farzi, BSc: *Human Milk Oligosaccharides: Natural Functional Foods for Fetus and Newborn*

Introduction: Human milk oligosaccharides (HMOs) are the third most abundant solid constituent in human milk, after lactose and lipids. HMOs stimulate the growth of preferably beneficial bacteria and might be the first prebiotics that humans are exposed to in life. Recently, the occurrence of HMOs has been reported in amniotic fluid. As a result of swallowing amniotic fluid, the fetus may come into direct contact with microorganisms already in utero.

Hypothesis: We hypothesize that HMOs in amniotic fluid can reach the fetal gut and can be found in meconium with profiles resembling those in amniotic fluid. We further hypothesize that the fetal gut has some capacity to metabolize certain HMOs. We thus aimed to 1) analyze excreted HMOs in meconium and to compare these HMO profiles with HMOs ingested via amniotic fluid and colostrum. We further aimed to 2) analyze HMOs in maternal milk and the corresponding infant stool specimens, to investigate the metabolic fate of HMOs passing the early colonized gut compared to the fetal/neonatal gut.

Methods: HMOs were isolated using chloroform/methanol extraction followed by deproteination using C18 columns and desalting with porous graphitic carbon columns. HMOs were labelled with the fluorescence tag 2-aminobezamide and separated by HPLC (Agilent 1200) on an amide-80 column using a linear gradient.

Results and Discussion: Our study showed that HMOs are present in meconium. Comparison of HMO patterns of meconium with corresponding amniotic fluid and colostrum samples showed several common HMO structures in all matrices. However, certain HMOs decreased significantly in meconium. Our results indicate that changes in abundances of individual HMOs might point to the activity of microbial enzymes. Compared to meconium, different HMOs were significantly decreased in stool, suggesting colonization of adapted bacterial microbiota under the influence of breast milk.

Conclusion: To our knowledge, this is the first report that HMOs appear in meconium. Comparison of meconium and corresponding amniotic fluid samples indicate that the fetal microbiome already commences in utero. Identifying HMOs in amniotic fluid as modulators in the development of a healthy gut microbiome could lay the foundation for future postnatal care of very preterm infants and the application of amniotic fluid as an alternative source of HMOs during the transition of from fetal to postnatal nutrition.

Andrea Stjepic, BSc: *Occurrence of bioactive amines in foods*

Bioactive amines are compounds prevalently occurring in animals, plants and humans but also in various types of food products and have physiological influences on the human body. One of them is the indoleamine melatonin generated in several intermediate stages from tryptophan. Biogenic amines such as spermidine or putrescine are produced, besides other processes, in decarboxylation reactions. In this master thesis, plant food was examined for the content of bioactive amines. Several foods were included in the analysis for which higher amine levels were found in the existing literature. Fresh products and fruits were selected based on seasonal availability. The amines melatonin, tyramine, tryptamine, spermidine, spermine, ß-phenethylamine, cadaverine and putrescine were analyzed with differences in the methods for melatonin. All measurements were carried out with HPLC using fluorescence detector, one method (derivatization with 9-Fluorenylmethoxycarbonyl chloride) included UV detection as

well. Methanol (100%) and water were used as solvents. The extraction of melatonin was performed using methanol as solvent. With this analysis method, the highest melatonin levels were observed in mustard seeds, particularly in black mustard seeds. However, no melatonin could be found in the fruits examined. Another method based on derivatization of melatonin with FMOC-Cl was carried out. The aim was to compare both procedures in regard to sensitivity and concentrations obtained but difficulties in identification of the melatonin peak lead to no results. The biogenic amines were derivatized with dansyl-chloride. In many food products, the amines were detected in low amounts below the limit of quantification. Putrescine was found in all foods except green pepper, whereas cadaverine was only detected in low traces in pumpkin seeds. Spermidine was present in all analyzed samples.

Adnan Fojnica, BSc: *Identification and characterization of the stability of hydrophobic cyclolinopeptides from flaxseed oil*

Flaxseed (linseed) is a cultivar of the spring flowering annual plant flax (Linum usitatissimum) from the Linaceae family. Derivatives of this plant are widely used as food and as health products. In recent years, cyclic peptides isolated from flaxseed and flaxseed oil, better known as cyclolinopeptides (CLPs), have attracted the attention of the scientific community due to their role in the inhibition of osteoclast differentiation or their antimalarial, immunosuppressive, and antitumor activities, as well as their prospects in nanotechnology and in the biomedical sector.

This study describes the detection, identification, and measurement of CLPs in samples obtained from nine different flaxseed oil manufacturers. For the first time, Q Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometry was used for CLP identification together with RP-HPLC. The routine analyses were performed using RP chromatography measuring the absorption spectra and fluorescence detection for identifying tryptophan-containing peptides using the native fluorescence of tryptophan.In addition, existing protocols used for CLP extraction were optimized and improved in a fast and cost-efficient way. For the first time, 12 CLPs were separated using methanol/water as the eluent with RP-HPLC. Finally, the stability and degradation of individual CLPs in the respective flaxseed oil were examined over a period of 60 days at different temperatures. The higher temperature was chosen since this might reflect the cooking practices, as flaxseed oil is not used for high temperature cooking. Using HPLC-MS, 15 CLPs were identified in total in the different flaxseed oils. The characterization of the peptides via HPLC-MS highlighted two types of CLPs pro-files with a substantial variation in the concentration and composition of CLPs per manufacturer, probably related to the plant cultivar. Among the observed CLPs, CLP-O, CLP-N, and CLP-B were the least stable, while CLP-C and CLP-A were the most stable peptides. However, it is important to highlight a gradual degradation of most of the examined CLPs over time, even at room temperature.

International cooperations

I. Cantalejo, Universidad de Navarra, Pamplona, Spain

K. Cejpek, VSCHT Prague, Czech Republic

Z. Cieserova, Food Research Institute, Bratislava, Slowakia

M. Mateus, H. Pinheiro, Instituto Superior Tecnico, Lisboa, Portugal

F. Pedreschi, Pontificia Universidad Catolica de Chile, Santiago, Chile

R. Swasti, Atma Jaya University, Yogyakarta, Indonesia

Talk at international conference

1. Murkovic, M.: *Chemical food safety in an increasing complex world.* IUFOST, Singapore.
2. Murkovic, M.: *Contaminants originating from heat processing of foods.* CEFood, Catez of Savi, Slovenia.

Publications

1. Wonisch, W., Stanger, O., Tatzber, F., Lindschinger, M., Murkovic, M., Cvirn, G.: *Stability of bioactive components in smoothies within an extended period of one year.* (2022) ***Food Bioscience*** 49, 101948. DOI: 10.1016/j.fbio.2022.101948
2. Fojnica, A., Leis, H.J., Murkovic, M.: *Identification and Characterization of the Stability of Hydrophobic Cyclolinopeptides From Flaxseed Oil.* (2022) ***Frontiers in Nutrition*** 9, 903611. DOI: 10.3389/fnut.2022.903611

Conferences / workshops organized

1. *Microalgae as foods of the future*, GÖCH, Graz
2. *Austrian food chemistry days 2022*, Graz
3. *Food chemistry updated*, on-line lecture series

Lectures and Laboratory Courses

Winter Semester 2020/21

Course no.	Title	Hours	Type	Lecturers
CHE.154_1UF	Biochemistry Lab Course I	5.33	LU	Team
CHE.155UF	Biochemistry II	1.5	VO	Macheroux P
CHE.191UF	Bioanalytics	2.25	VO	Klimant I, Winkler A
CHE.192UF	Biochemistry Lab Course II	4	LU	Bijelic A, Wallner S
CHE.200_FUF	Project Lab (Bachelor)	5	LU	PIs
CHE.210_FUF	Project Work to the Bachelor Thesis	0.5	SE	PIs
CHE.890UF	Food Biotechnology	1.33	VO	Murkovic M
CHE.892UF	Enzymatic and Microbial Food Processing	2	VO	Murkovic M
MOL.101UB	Introduction to Bachelor Study	1	SE	Macheroux P
MOL.606_1UF	Bachelor Thesis	1	SE	PIs
MOL.832_1UF	Project Laboratory	9	LU	Team
MOL.844_1UF	Journal Club Biochemistry and Molecular Biomedicine	1.5	SE	Team
MOL.845_1UF	Master Thesis Seminar	2	SE	PIs
MOL.855UF	Molecular Physiology	2	VO	Lorber B, Macheroux P
MOL.881UB	Biophysical Methods	2	SE	Winkler A, …
MOL.882_1UF	Profession-oriented Research Practices	2	PV	Macheroux P
MOL.933UF	Food Biotechnology	1.3	VO	Murkovic M
MOL.936UF	Food Biotechnology	4	LU	Murkovic M, Leitner E
MOL.959UF	Enzymatic and Microbial Food Processing	2	VO	Murkovic M
MOL.961UF	Food Chemistry and Technology II	2	VO	Murkovic M
648.003	Molecular Enzymology I	2	PV	Macheroux P
648.007	Graduate Seminar 1	1	SE	Team
648.009	Scientific Colloquium for Graduate Students 1	1	SE	Team
648.021	Structural Biochemistry I	2	PV	Winkler A
648.030	Soft Skills Doctoral School Molecular Biomedical Sciences and Biotechnology	1	SE	Macheroux P
649.027	Chemical Reactions in Foods I	2	PV	Murkovic M

VO: Lecture Course; LU Lab Course; SE Seminar; PV: Privatissimum

Lectures and Laboratory Courses

Summer Semester 2021

Course no.	Title	Hours	Type	Lecturers
CHE.147UF	Biochemistry I	3.75	VO	Macheroux P
CHE.193UF	Molecular Biology Laboratory Course	3	LU	Kerschbaumer B, Wallner S
CHE.194UF	Seminar for Molecular Biology Laboratory Course	1	SE	Kerschbaumer B, Wallner S
CHE.200_FUF	Project Laboratory Chemistry (Bachelor)	5	SE	Macheroux P, Murkovic M, Oberdorfer G, Winkler A
CHE.210_FUF	Project Work to the Bachelor Thesis	0.5	SE	Macheroux P, Murkovic M, Oberdorfer G, Winkler A
MOL.406UF	Methods in Immunology	2	VO	Oberdorfer G
MOL.407UF	Methods in Immunology	2	LU	Team
MOL.606_1UF	Bachelor Thesis	1	SE	Team
MOL.832_1UF	Project Laboratory	9	LU	Team
MOL.844_1UF	Journal Club Biochemistry and Molecular Biomedicine	1.5	SE	Team
MOL.845_1UF	Seminar for Undergraduate Students	2	SE	Team
MOL.880UF	Molecular Enzymology	2	VO	Gruber K, Macheroux P, Nidetzky B
MOL.886UB	Biophysical Methods	3	LU	Winkler A, Oberdorfer G, […]
MOL.969UF	Integrative Structural Biochemistry	2	VO	Winkler A, Madl T, Gruber C
MOL.970UF	Protein Design	2	VO	Oberdorfer G
648.004	Molecular Enzymology II	2	PV	Macheroux P
648.006	Introduction to Biochemistry	2	VO	Wallner S
648.008	Graduate Seminar 2	1	SE	Team
648.010	Scientific Colloquium for Graduate Students 2	1	SE	Team
648.012	Frontiers in Integrative Structural Biology	1	SE	Macheroux P, Winkler A, Oberdorfer G, …
648.016	Chemical Reactions in Foods II	2	PV	Murkovic M
648.022	Structural Biochemistry II	2	PV	Winkler A

VO: Lecture Course; LU Lab Course; SE Seminar; PV: Privatissimum