July 2-6, 2017

Groningen, The Netherlands

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The 19th International Symposium on Flavins and Flavoproteins will be held in Groningen, The Netherlands. The symposium will highlight recent developments in the field of flavin-dependent proteins and processes. Except for various lectures by invited experts in the field, the program will include oral presentations (selected based on submitted abstracts), young investigator talks, poster pitch talks, and posters. The vibrancy of the field of flavin-based chemistry and flavoenzymology will be highlighted in sessions on:

Newly discovered flavoproteins / Flavoenzyme engineering / Flavin-based chemistry Flavoenzyme mechanisms & structures / Flavoproteins & light / Flavoenzymes & health / Flavoenzymes & industrial applications

Scientific Committee

Pimchai Chaiyen (Mahidol University, Thailand)
Brian Crane (Cornell University, USA)
Kiyoshi Fukui (Tokushima University, Japan)
Giovanni Gadda (Georgia State University, USA)
Peter Macheroux (Graz University of Technology, Austria)
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Milagros Medina (University of Zaragoza, Spain)
Bruce Palfey (University of Michigan, USA)
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WIFI

Free wifi is available at the conference venue.

Program

Sunday, July 2, 2017 Der Aa-kerk

14:00		Welcome and registration
16:00		Opening
		Session 1: Introductory Lecture
		Chair: **Willem van Berkel** (Wageningen University & Research)
16:15	IL1	Opening lecture: **Sandro Ghisla** (University of Konstanz) *Flavins, a look back to the possible origins, and a glimpse forward*
		Session 2: Flavin-based Chemistry
		Chair: **Willem van Berkel** (Wageningen University & Research)
17:00-17.45	IL2	**Kurt Faber** (University of Graz) *Flavin-based redox-neutral biotransformations*
17.45-18.15		*Break – drinks and snacks*
18:15-18.45	L1	**Radek Cibulka** (University of Chemistry and Technology, Prague) *Rational design of flavins for organic photoredox catalysis: from photolyase models to effective tool in organic synthesis*
18:45-19.15	L2	**Erik Schleicher** (University of Freiburg) *Radicals and radical pairs in flavoproteins*
19.15-19.45	L3	**Robert Stanley** (Temple University, Philadelphia) *The optical absorption spectrum of reduced flavin is more complex than we knew: why it matters*
20.00-22.00		**Welcome reception/walking dinner**

The reception will be offered to you by the University of Groningen, the Municipality of Groningen and the Province of Groningen

Monday, July 3, 2017 Groninger Forum

08:00-08.55		Registration and coffee/tea
08.55-09.00		Opening
		Session 3: Newly Discovered Flavoenzymes I
		Chair: **Bruce Palfey** (University of Michigan, Ann Arbor)
09.00-09.45	IL3	**Davis Leys** (University of Manchester) *The UbiX-UbiD system: biochemistry of prenylated flavin*
09.45-10.15	L4	**Karl Payne** (University of Manchester) *Structural and mechanistic insights into prFMN dependent aromatic reversible (de)carboxylases*
10.15-10.45	L5	**Valentino Konjik** (Mannheim University of Applied Sciences) *RosB is the key enzyme of roseoflavin biosynthesis*
10.45-11.15		*Coffee/tea break*
		Session 4: Flavoenzyme Structures and Mechanisms I
		Chair: **Matthias Mack** (Mannheim University of Applied Sciences)
11:15-12.00	IL4	**Pimchai Chayen** (Vidyasirimedhi Institute of Science and Technology, Rayong) *Beyond monooxygenation by flavin-dependent enzymes*
12.00-12.30	L6	**Thomas Lautier** (Institut National des Sciences Appliquées de Toulouse) *Cytochrome P450 reductase chimera: turn around the hinge*
12:30-13:00		**Poster Pitch talks (7x)**
		Felipe Calil (*University of São Paulo*) *Inhibition studies of the dihydroorotate dehydrogenase from S. mansoni using antimalarial drugs repositioning* **Litavadee Chuaboon** (Mahidol University, Bangkok) *Engineering of pyranose 2-oxidase for expanding substrate utilization* **Marina Toplak** (Technical University of Graz) *The origin of the berberine bridge enzyme-like protein family in plants* **Simon Ernst** (University of Münster) *PqsL, an arylamine-hydroxylating monooxygenase involved in 2-heptyl-4-hydroxyquinoline-N-oxide biosynthesis* ***Filippo Fiorentini*** *(University of Pavia)* *Human FMO5: discovery of Baeyer-Villiger reactions in human oxidative metabolism* ***Eric Gädke*** *(TU Bergakademie Freiberg)* *Inhibition of 'thermophilic-like' ene-reductases by an N-terminal cysteine* ***Andreea Iorgu*** *(University of Manchester)* *NMR and stopped-flow studies pinpoint key residues involved in enzymatic hydride transfer in PETNR flavoenzyme*
13.00-14.00		*Lunch*
14.00-15.30		*Poster session + coffee/tea*
15.30-16.00		*Drinks and snacks*

Session 5: Flavoenzyme Engineering

Chair: **Marco Fraaije** (University of Groningen)

16:00-16.45 L5 **Tobias Erb** (Max Planck Institute for Terrestrial Microbiology, Marburg)
CETCH me if you can: bringing inorganic carbon into life with synthetic CO_2 fixation and flavoenzyme engineering

16.45 – 17.15 L7 **David Ackerley** (Victoria University, Wellington)
Discovery, characterisation and engineering of bacterial nitroreductases

17.15–17.45 L8 **Steven Rokita** (Johns Hopkins University, Baltimore)
Facile interconversion of a FMN-dependent dehalogenase and nitroreductase

Session 6: Young Investigators Talks

17:45 YIT1 **Tobias Hedison** (University of Manchester)
Real-time analysis of conformational control in electron transfer reactions of diflavin oxidoreductases

18.05 YIT2 **Kelsey Kean** (Oregon State University, Corvallis)
High resolution studies of hydride transfer in the ferredoxin-$NADP^+$ reductase superfamily

18.25 YIT3 **John Robbins** (Georgia State University, Altanta)
Formate oxidase (FOX) activity is governed by autocatalytic formation of 8-formyl FAD exhibiting unusual properties

18.45 Closure

Tuesday, July 4, 2017 Groninger Forum

08:30-08.55 Welcome and coffee/tea
08.55-09.00 Opening

Session 7: Flavoproteins & Health I

Chair: **Milagros Medina** (University of Zaragoza)

09.00 – 09.45 IL6 **Djemel Hamdane** (Laboratoire de Chimie des Processus Biologiques, Paris)
Flavin-dependent epitranscriptomic: when flavoenzymes regulate the translation

09.45 – 10.15 L9 **Jung-Ja Kim** (Medical Centre of Wisconsin, Milwaukee)
Electron transfer flavoprotein goes moonlighting

10.15 – 10.45 L10 **Marie Antonietta Vanoni** (University of Milan)
Human MICAL-1 the multidomain flavoenzyme participating to actin cytoskeleton dynamics

10.45 – 11.15 *Coffee/tea break*

Session 8: Flavoproteins & Light

Chair: **Brian Crane** (Cornell University, Ithaca)

11:15 – 12.00 IL7 **John Kennis** (Free University Amsterdam)
Photoactivation mechanisms of flavin-binding photoreceptors

12.00 – 12.30 L11 **Brian Crane** (Cornell University, Ithaca)
Light and redox sensing by flavoproteins

12.30 – 13.00		**Poster Pitch talks (8x)**
		Sami Ullah Khan (Graz University of Technology) *The interaction of human dimethylglycine dehydrogenase with electron transferring flavoprotein* **Jittima Phonbuppha** (Mahidol University, Bangkok) *Construction of eukaryotic bioreporter systems based on bacterial luciferase* **Piero Leone** (University of Bari) *Functional characterization and homology modeling of a novel monofunctional isoform of human FAD synthase* **Morten van Schie** (Delft University of Technology) *Kinetics of the light-driven reduction of 5-deaza riboflavins* **Kiyofumi Takaba** (Kyoto University) *The role of valence electrons of FAD on the directivity of electron transfer in NADH-cytochrome b_5 reductase* **Duangthip Trisvirat** (Mahidol University, Bangkok) *Kinetics and product analysis of oxidase and monooxygenase activities of L-amino acid oxidase and monooxygenase* **Andreas Winkler** (Graz University of Technology) *Molecular mechanisms of light regulation in LOV-diguanylate cyclases - implications for sensor-effector modularity* **Hiromi Yoshida** (Kagawa University) *X-ray structure analysis of fructosyl peptide oxidases to elucidate the residues responsible for the oxidative half-reaction*
13.00-14.00		*Lunch*
14.00-15.30		*Poster session + coffee/tea*
15.30-16.00		*Drinks and snacks*
		Session 9: Flavoenzymes & Biotechnology
		Chair: **Nigel Scrutton** (University of Manchester)
16:00 – 16.45	IL8	**Yasuhisa Asano** (Toyama Prefectural University) *Use of R-amine oxidase evolved from D-amino acid oxidase for chiral (S)-amine synthesis and oxidative cyanation reaction*
16.45 – 17.15	L12	**Sheila Sadeghi** (University of Torino) *Biotechnological application of human FMO3: an electrochemical biochip for drug metabolism*
17.15 – 17.45	L13	**Selin Kara** (Hamburg University of Technology) *Enzymatic redox-neutral convergent cascade for lactonizations*
		Session 10: Young Investigators Talks
17:45-18.05	YIT4	**Laura Mascotti** (Universidad Nacional de San Luis) *On the evolution of flavin-dependent enzymes: retracing the steps*
18.05-18.25	YIT5	**Hafna Ahmed** (Australian National University, Canberra) *Structure, function and evolution of F420H2 utilizing flavin/deazaflavin oxidoreductases (FDORs) in Mycobacteria*
18.25-18.45	YIT6	**Mieke Huijbers** (Wageningen University & Research) *Trimming proline dehydrogenase: protein and cofactor minimization*
18.45		Closure

Wednesday, July 5, 2017 Groninger Forum

08:30 – 08.55		Welcome and coffee/tea
08.55 – 09.00		Opening

Session 11: Newly Discovered Flavoenzymes II

Chair: **Kiyoshi Fukui** (Tokushima University)

09.00 – 09.45	IL9	**Robin Teufel** (University of Freiburg) *The structural prerequisites for enzymatic flavin-N5-oxide formation*
09.45 – 10.15	L14	**Margaret Ahmad** (University of Paris VI) *Cryptochromes and perception of electromagnetic fields: role of the redox photocycle and potential signaling mechanisms*
10.15 – 10.45	L15	**Thomas Heine** (Technical University Bergakademie Freiberg) *Enantioselectivtiy of styrene monooxygenases towards sulfides is defined by a tyrosine in the active site*
10.45 – 11.15		*Coffee/tea break*

Session 12: Flavoenzymes & Biotechnology II

Chair: **Pimchai Chaiyen** (Vidyasirimedhi Institute of Science and Technology, Rayong)

11:15 – 12.00	IL10	**Gianluca Molla** (University of Insubria) *Playing on both sides of the mirror: amino acid oxidases as versatile tools for chiral biocatalysis*
12.00 – 12.30	L16	**Sébastien Willot** (Technical University Delft) *A light-driven cascade to promote peroxygenase catalyzed reactions*

Session 13: Young Investigators Talks

12.30	YIT7	**Bastian Daniel** (Graz University of Technology) *Berberine bridge enzyme-like proteins: from characterization to application*
12.50	YIT8	**Daniel Kracher** (BOKU University Vienna) *Fungal flavoenzymes involved in cellulose degradation*
13.10	YIT9	**Wolf-Dieter Lienhart** (Graz University of Technology) *Drug development for cancer treatment and prevention targeting the human NAD(P)H:quinone oxidoreductase 1*
13.30		*Lunch and Free time / Excursions* ***Visit to Hooghoudt**: a bus to Hooghoudt will leave at 14.30* ***Walking Tour**: you will be guided to the center of Groningen*

Thursday, July 6, 2017 Groninger Forum

08:30 – 08.55		Welcome and coffee/tea
08.55 – 09.00		Opening

Session 14: Flavoenzyme Engineering II

Chair: **Giovanni Gadda** (Georgia State University, Atlanta)

09.00 – 09.45	IL11	**Elvira Romero** (University of Groningen) *Tuning the regioselectivity of a robust cyclohexanone monooxygenase by structure-inspired enzyme engineering*
09.45 – 10.15	L17	**Gudrun Gygli** (Wageningen University) *The ins and outs of vanillyl alcohol oxidase: identification of ligand migration paths*
10.15 – 10.45	L18	**Dennis Stuehr** (Cleveland Clinic) *Restricting FMN domain freedom and its impact on electron transfer and catalysis in the NO synthase flavoprotein*
10.45 – 11.15		*Coffee/tea break*

Session 15: Flavoproteins & Health II

Chair: **Willem van Berkel** (Wageningen University & Research)

11:15 – 12.00	IL12	**Maria Barile** (University of Bari) *Unraveling the role of riboflavin and its derived cofactors in health and diseases*
12.00 – 12.30	L19	**Patricia Ferreira** (Universidad de Zaragoza) *Human apoptosis inducing factor: from its molecular mechanism to its functional and pathological significance*
12.30 – 13.00	L20	**Francesca Magnani** (University of Pavia) *Crystal structures and atomic model of NADPH oxidase*
13.00 – 14.00		*Lunch / Scientific Committee meeting*

Session 16: Flavoenzyme Structures and Mechanisms II

Chair: **Peter Macheroux** (Graz University of Technology)

14:00 – 14.45	IL13	**Giovanni Gadda** (Georgia State University, Atlanta) *The biochemistry and biophysics of D-arginine dehydrogenase from Pseudomonas aeruginosa*
14.45 – 15.15	L21	**Hans-Petter Hersleth** (University of Oslo) *Activation of the class Ib ribonucleotide reductase by a flavin network in Bacillus cereus*
15.15 – 15.45	L22	**Milagros Medina** (University of Zaragoza) *Kinetics and thermodynamics in ligands binding as FMN production determinants in bifunctional FAD synthetases*
15.45 – 16.15	L23	**Anne-Frances Miller** (University of Kentucky, Lexington) *Ultrafast kinetic studies reveal effects of electron transfer and charge recombination on the lifetime of flavin semiquinone*

16.15 – 16.45	*Coffee/tea break*
16:45 – 17.45	General discussion: the future of flavoprotein research
17.45 – 18.30	**Vince Massey Awards & Poster Awards**
18.30	Closure
20.00 – 22.00	**Conference Dinner and Dinner Lecture - Der Aa-kerk, Akerkhof 2**
IL14	**Russ Hille** (University of California, Riverside) *Flavins, Flavoproteins and Flavinologists*

Invited Speakers & Lecture Abstracts

Flavins, a look back to the possible origins, and a glimpse forward

Sandro Ghisla

University of Konstanz, Konstanz, Germany

Keywords: Flavin, coenzyme, origin, role.

The flavin cofactor has a wide distribution in biological systems, it occurs in a variety of modifications, and it is characterized by an extreme versatility in its functions. This brings up questions about its origins and its possible role(s) in the emergence of life. Flavins (and pterins) are analogs of guanine and might have emerged concomitantly with, or just by chemical conversion from the latter[1]. They have, in their FAD form, been termed[1] "*fossil of the RNA World, … whose activity has been harnessed for myriad functions of life from metabolism to DNA repair*".

P. Chandler writes in "Evolution of the coenzymes"[2]: … many coenzymes are nucleotides or heterocyclic bases which could be derived from nucleotides (and) could be seen as a vestige of the existence of a primordial stage prior to the existence of proteins during which biological catalysis and metabolic pathways had been mediated by coenzymes". Flavins might thus have had a crucial role in the bioenergetics required by primordial life, such as in the much-discussed "RNA world".

In addition it is remarkable that flavins are cofactors in enzymes participating in the biogenesis of other (presumably "younger") coenzymes and of important biological key molecules, this being in line with their antique origin. It is thus reasonable to assume that life has taken advantage of the versatility of flavins at a very early stage in evolution. Some of these aspects will be discussed, also in view of implications for future research in the field.

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1 K. Van Nguyen, C. J. Burrows, JACS, 2011, 14586-14589; ACCOUNTS OF CHEMICAL RESEARCH Vol. 45, 12, 2012, 2151–2159.

2 Vol. III. Biochemistry, 149–157, 1963.

Flavin-based redox-neutral biotransformations

K. Durchschein,[1] S. M. Glueck,[1] M. Hall,[1] T. Pavkov-Keller,[2] S. Payer,[1]
G. Steinkellner,[2] N. Turrini,[1] C. K. Winkler,[1] C. Wuensch,[1] K. Faber [1]

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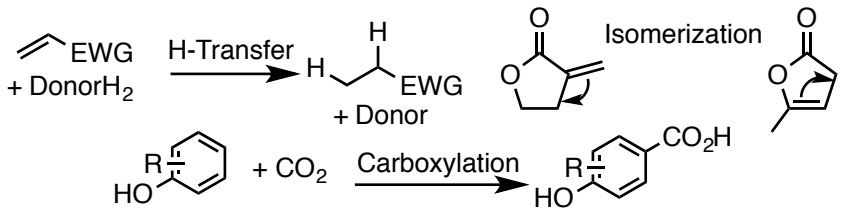
Keywords: Alkene reduction, alkene isomerization, carboxylation, prenylated FMN.

During the past decade, flavoproteins became widely recognized for their ability to catalyze a broad variety of synthetically useful biotransformations, which are difficult to perform using chemical methodology.1 Besides the well-known asymmetric bioreduction of alkenes bearing an electron-withdrawing (activating) substituent (EWG) at the expense of an external hydride donor via NAD(P)H,2 nicotinamide-independent bioreduction can be achieved through *inter*molecular redox-neutral H-transfer.3 This concept was extended to the *intra*molecular isomerization of *exo*-methylene γ-butyrolactones yielding the corresponding *endo*-isomers in presence of cat. NAD(P)H.4 Surprisingly, also non-activated β,γ-unsaturated α-Angelica lactone could be isomerized to the conjugated β-analog in the absence of nicotinamide, presumably via acid-base catalysis.5



The (redox-neutral) carboxylation of electron-rich (hetero)aromatics yields the corresponding carboxylic acids and represents a biocatalytic equivalent to the Kolbe-Schmitt-process. Regioselective *o*-carboxylation is achieved by Lewis-acid (Zn2+) depending carboxylases,6 while the β-carboxylation of hydroxystyrenes is mediated by phenolic acid decarboxylases acting via an Arg-Tyr pair.7 In search for a regio-complementary (ATP-independent) *p*-carboxylase, we identified 3,4-dihydroxybenzoate decarboxylase from *Enterobacter cloacae*, which requires a prenylated FMN (prFMN) as cofactor for activity, which was only recently identified.8

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Rational design of flavins for organic photoredox catalysis: from photolyase models to effective tool in organic synthesis

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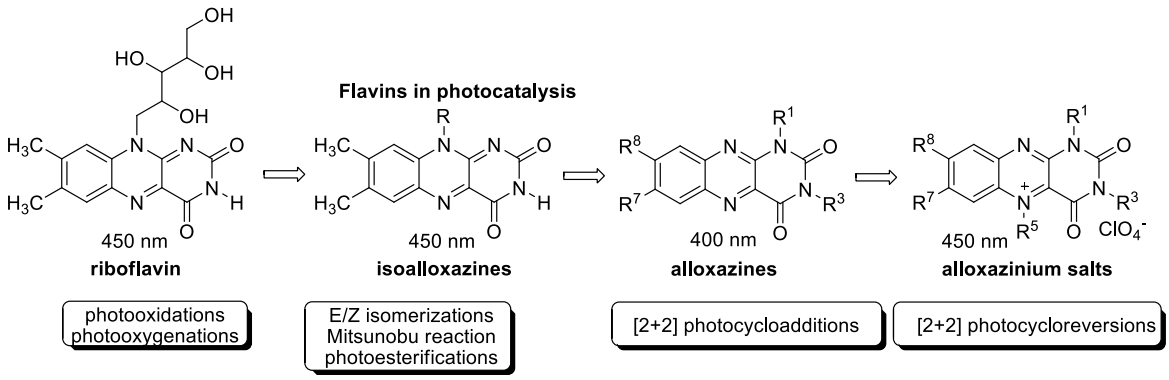
Keywords: Photocatalysis, Mitsunobu esterification, Photooxidation, Cycloreversion.

Photoexcitation allows chemical transformations that are not accessible by conventional methods. Use of visible light combined with a photocatalyst even improves classical photochemical methodologies avoiding side reactions of functionalities sensitive to UV light and making photochemistry available for most laboratories. Nowadays, photo-redox catalysis with organic photocatalysts[1] (organic photo-redox catalysis, photoorganocatalysis) is of a growing interest because of low costs of organic dyes and different reactions that they are able to mediate thus expanding the boundaries of organic synthesis.

One of the most prominent natural chromophores is represented by flavin cofactors which are involved in several light-dependent processes, for example, in light generation by bacterial luciferase, in plant phototropism and in photolyases in the cleavage of cyclobutane-pyrimidine dimers to repair DNA damage. Due to interesting photochemical properties, flavins have been tested in photocatalysis; nevertheless, their applications, except of photolyase models,[2] have been for a long time limited to photooxidations of benzylic derivatives.[3] In the presentation, recent flavin-based photocatalytic procedures will be discussed, especially visible light [2+2] photocycloaddition[4] and photocycloreversion,[5] photooxygenation,[6] photocatalytic Mitsunobu reaction,[7] esterification and Apple reaction. Special attention will be paid to rational design of flavin photocatalysts (see Scheme for illustration).



Authors thank the Czech Science Foundation (grant No 16-09436S) for financial support.

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4 Mojr, V., Svobodová, E., Straková, K., Neveselý, T., Chudoba, J., Dvořáková, H., and Cibulka, R. (2015) Tailoring flavins for visible light photocatalysis: organocatalytic [2+2] cycloadditions mediated by a flavin derivative and visible light. Chem. Commun. 51, 12036-12039.

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7 März, M., Chudoba, J., Kohout, M., and Cibulka, R. (2017) Photocatalytic esterification under Mitsunobu reaction conditions mediated by flavin and visible light. Org Biomol Chem 15, 1970-1975.

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Radicals and radical pairs in flavoproteins

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Keywords: flavin radicals, flavoproteins, cryptochromes, EPR spectroscopy

Flavin semiquinones are common intermediate redox states in flavoproteins and hence, detailed knowledge of their chemical nature is required for a full understanding of their versatility in catalysis [1]. In this contribution, we use a number of (time-resolved) molecular spectroscopy methods to explore flavin radicals in various protein and non-protein environments.

Three examples will be presented: First, we were able to stabilize anionic and neutral flavin radicals in aqueous solutions and characterize both states using electron paramagnetic resonance (EPR) methods. Second, the radical state of a recently discovered flavin intermediate in monooxygenases, the flavin-N(5)-oxide, was analyzed in detail. The resulting electron-spin density distribution lead to a classification in between a “normal” flavin cofactor radical and a well-established nitroxide spin label. Finally, short-lived paramagnetic intermediates of flavin-dependent cryptochrome photo- and magnetoreceptors were characterized by time-resolved EPR methods of all flavors [2]. Here, details in electron-transfer pathways could be revealed [3], which have direct impact on the ability of insect cryptochromes to perceive magnetic fields [4].

The work presented has been performed in collaboration with:

A. Bacher, B. Illarionov, M. Fischer (Hamburg University); P. J. Hore, C. Timmel and S. Mackenzie (Oxford University); .R. Batcha and R. Teufel (Freiburg University)

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2 Paulus et al., (2015) „Spectroscopic characterization of radicals and radical pairs in fruit fly cryptochrome – protonated and nonprotonated flavin radical-states” FEBS Journal 282, 3175

3 Nohr et al., (2016) „Extended Electron-Transfer in Animal Cryptochromes Mediated by a Tetrad of Aromatic Amino Acids“, Biophys J 111, 301.

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The optical absorption spectrum of reduced flavin is more complex than we knew: why it matters

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Keywords: photobiology, spectroscopy, Stark spectroscopy, TD-DFT

Chromophoric biomolecules are exploited as reporters of a diverse set of phenomena, acting as internal distance monitors, environment and redox sensors, and endogenous imaging probes. In the case of flavins, photoinduced electron transfer and photochemistry are also important outcomes of the absorption of light. In either case, it is of the greatest importance to have a precise knowledge of the direction(s) of the absorption transition dipole moment(s) (TDMs) in the molecular frame of reference for the absorption band in question. The directions of the TDMs in oxidized and semiquinone flavins were characterized decades ago,[1] and the details of charge redistribution in these forms have also been studied by Stark spectroscopy.[2] The electronic structure of the fully reduced hydroquinone anionic state, FlH^-, however, has been the subject of untested estimates of the number and direction of TDMs therein, as well the electronic structure changes that occur upon light absorption. Here we have used Stark spectroscopy to measure the magnitude and direction of charge redistribution in FlH^- upon optical excitation. These data were analyzed using TD-DFT calculations. The results show unequivocally that not one but two nearly orientation-degenerate electronic transitions are required to explain the 340–500 nm absorption spectral range, demolishing the commonly held assumption of a single transition. The difference dipole moments for these states show that electron density shifts toward the xylene ring for both transitions. These measurements force a reappraisal of previous studies that have used erroneous assumptions and unsubstantiated estimates of these quantities. The results put future optical studies of reduced flavins/flavoproteins on a firm photophysical footing.

References

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[2] Pauszek, R. F., Kodali, G., and Stanley, R. J. (2013) Stark Spectroscopy of Flavins, In Methods in Molecular Biology (E, S., and S, W., Eds.), Humana Press USA, New York.

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The UbiX-UbiD system: biochemistry of prenylated flavin

David Leys

The UbiX-UbiD system consists of the flavin prenyltransferase UbiX that produces prenylated FMN that serves as the cofactor for the (de)carboxylase UbiD. Recent developments have provided structural insights into the mechanism of both enzymes, detailing unusual chemistry in each case. The proposed reversible 1,3 dipolar cycloaddition between the cofactor and substrate serves as a model to explain many of the key UbiD family features. However, considerable variation exists in the many branches of the UbiD family tree. An overview of this system as well as new data recently obtained in our group will be presented.

Structural and mechanistic insights into prFMN dependent aromatic reversible (De)carboxylases

Karl A. P. Payne[1], Stephen A. Marshall[1], Sam S. Bailey[1], Karl Fisher[1], Nigel S. Scrutton[1], David Leys[1]

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Keywords: prFMN, Modified Flavin, Decarboxylase, Protein Structure,

The UbiD/Fdc1 family are a wide spread and diverse family of enzymes capable of catalyzing the reversible (de)carboxylation of aromatic or unsaturated aliphatic substrates. These enzymes were recently discovered to be dependent on a novel prenylated form of FMN (termed prFMN) where a prenyl group is attached to the N5 and C6 positions of the isoalloxazine ring system such as to create a 4th non-aromatic ring[1]. In the case of the fungal Fdc1 enzymes prFMN has been shown to support (de)carboxylation via 1,3-dipolar cycloaddition between the unsaturated aliphatic substrate (a dipolarophile) and the azomethine ylide (a well known dipole) of the modified cofactor[2]. Whilst this mechanism has been generally accepted for prFMN dependent enzymes that act on aliphatic substrates, the involvement of 1,3-dipolar cycloaddition in the (de)carboxylation of aromatic substrates remains controversial as it would require transient dearomatisation of the ring system. Here we report the application of a number of structural, biophysical and biochemical techniques to further elucidate the mechanism of these enzymes.

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RosB is the key enzyme of roseoflavin biosynthesis

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Keywords: Roseoflavin, Flavodoxin, AFP synthase, *Streptomyces davawensis*.

The antibiotic roseoflavin (RoF) is the only known natural riboflavin (vitamin B_2) analogue and is active against gram-positive bacteria. RoF is produced by *Streptomyces cinnabarinus* and *Streptomyces davawensis* and can be considered to be an "antivitamin". In RoF biosynthesis the C8 methyl of the precursor riboflavin is replaced by a dimethyl amino group whereby 8-demethyl-8-amino-riboflavin (AF) was postulated to be an intermediate. The first discovered enzyme of roseoflavin biosynthesis was the *S*-adenosyl methionine (SAM) dependent dimethyltransferase RosA which converts AF to RoF. Previous experiments suggested that a single enzyme (RosB) was responsible for the formation of AF. However, when recombinant RosB was tested in an assay mixture containing riboflavin-5'-phosphate (RP) the formation of the predicted final reaction product AF or 8-demethyl-8-aminoriboflavin-5'-phosphate (AFP) was not observed. Instead the compound 8-demethyl-8-formyl-riboflavin-5'-phosphate (HOC-RP) was detected, probably an intermediate of the RosB reaction. How the formyl-group of HOC-RP was replaced by an amino group to give AF or 8-demethyl-8-amino-riboflavin-5'-phosphate (AFP) remained unclear.

The present work was initiated to investigate the predicted oxidation of HOC-RP to 8-demethyl-8-carboxyl-riboflavin-5'-phosphate (HOOC-RP), to identify the amino group donor of the RosB reaction and to shed light on the reaction mechanism of the multi-step enzyme RosB. Heterologous gene expression and biochemical studies revealed that RosB accepts only RP as a substrate and not riboflavin. RosB activity depends on the presence of O_2, thiamine and the amino group donor glutamate. HOOC-RP was found to be an (additional) intermediate of the RosB reaction. The crystal structure of RosB was solved with bound AFP (1.7 Å) and HOC-RP (2.0 Å). RosB is composed of four flavodoxin-like subunits which have been upgraded with specific extensions and a unique C-terminal arm. Structure-based active site analysis was complemented by mutational and isotope-based mass-spectrometric data to propose an enzymatic mechanism.

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Beyond monooxygenation by flavin-dependent enzymes

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Flavin-dependent enzymes are among the most versatile redox catalysts found in nature. We have investigated reaction mechanisms of several two-component flavin-dependent monooxygenases which use C4a-hydroperoxyflavin as a reactive intermediate for catalyzing oxygenation. These enzymes consist of a reductase component which generates reduced flavin as a product and an oxygenase component which utilizes the reduced flavin as a substrate. Many flavin-dependent hydroxylases can also catalyze additional reactions beyond the hydroxylation alone. We will discuss four examples of flavin-dependent monooxygenases. The first system is HadA which is a dechlorinase that can catalyze hydroxylation in conjunction with dechlorination. HadA can convert various chlorinated phenols, commonly used agrochemicals that cause environmental problems, into less toxic materials that can be assimilated by microbes. The enzyme is thus useful for future development in detoxification and biorefinery applications. The second system is tryptophan 6-halogenase that catalyzes regio-specific chlorination of tryptophan. This enzyme is very useful for biocatalytic applications. The third system is *p*-hydroxyphenylacetate (HPA) 3-hydroxylase (HPAH), an enzyme that can incorporate a hydroxyl group regio-specifically into phenolic compounds. We found that HPAH can convert *p*-coumaric acid (lignin-derived compound) into 3,4,5-trihydroxycinnamic acid (3,4,5-THCA), a strong antioxidant that is potentially useful as medicinal agents. We also engineered HPAH and obtained enzyme variants that can be used for synthesizing dopamine from tyramine and hydroxylation of aniline derivatives. The last system is bacterial luciferase that can catalyze light emitting reaction. This enzyme can be applied in bioreporter applications.

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Cytochrome P450 reductase chimera: turn around the hinge

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Keywords: NADPH-cytochrome P450 reductase, salt effect, domains dynamic, hinge domain.

NADPH-cytochrome P450 reductase (CPR) is the redox partner of the cytochrome P450 enzymes, involved in sterol metabolism and xenobiotic detoxification. The CPR contains a membrane anchored FMN domain linked to a FAD domain by a hinge region and a connecting domain (Gutierrez *et al.*, 2003). The CPR oscillates between a compact conformation positioning FMN and FAD in a suitable orientation and distance for electron transfer (locked state) and a series of open conformations (unlocked state) that probably support the reduction of external acceptors. This conformational equilibrium has been shown to be highly dependent on ionic strength, strengthening the hypothesis of the presence of salt interactions at the interface between the FMN and connecting FAD domains (Frances *et al.*, 2015). In the present study, six single mutants and two double mutants of the human CPR targeting the hinge segment have been constructed and their biochemical behaviors analyzed in function of the ionic strength. Reduction of cytochrome c, an unnatural substrate, at various salt concentrations has been quantified in these 8 mutants, introduced either in the soluble or membrane-bound forms of human CPR. All mutants were found active with various efficiencies and a continuum in their optimum salt concentration. The presence of the membrane does not deeply modify the ionic strength profiles, even if some key differences appear in regard of the soluble form. The weight of each position has been implemented in a view of a global open end zip dynamic system between the FAD-FMN domains. This work illustrates that both flexibility and ionic interactions are both controlling the function of the hinge.

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CETCH me if you can: bringing inorganic carbon into life with synthetic CO2 fixation and flavoenzyme engineering

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Carbon dioxide (CO2) is a potent greenhouse gas that is a critical factor in global warming. At the same time atmospheric CO2 is a cheap and ubiquitous carbon source. Yet, synthetic chemistry lacks suitable catalysts to functionalize atmospheric CO2, emphasizing the need to understand and exploit the CO2 mechanisms offered by Nature.

In my talk we will discuss the evolution and limitation of naturally existing CO2 fixing enzymes and pathways. I will present strategies for the engineering and design of artificial CO2 fixation reactions and pathways (*Peter et al. 2015*), and outline how these artificial pathways can be realized and further optimized to create synthetic CO_2 fixation modules.

An example for such a synthetic CO_2 fixation module is the CETCH cycle (*Schwander et al. 2016*). The CETCH cycle is an *in vitro*-reaction network of 17 enzymes that was established with enzymes originating from nine different organisms of all three domains of life. A key in establishing the CETCH cycle was the rational engineering and redesign of two flavoenzymes to catalyze new reactions. With these engineered flavoenzymes, the CETCH cycle (in its version 5.4) converts CO_2 into organic molecules at a rate of 5 nanomoles of CO_2 per minute per milligram of protein. This is slightly faster than the Calvin cycle under comparable conditions and notably at 20% less energy per CO_2 fixed.

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Discovery, characterisation and engineering of bacterial nitroreductases

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Keywords: Directed evolution; Enzyme-prodrug therapy; Targeted cell ablation; Metagenomic biodiscovery

Bacterial nitroreductases are NAD(P)H-dependent oxidoreductases (generally homodimeric and FMN-binding) that can catalyse the 4- or 6-electron reduction of nitro groups on aromatic rings. This results in a profound electronic shift that can dramatically alter the properties of the molecule as a whole, e.g. activating latent cytotoxins, or detoxifying certain pollutants or antibiotics. We have exploited these properties and the characteristic promiscuity of these enzymes to develop useful tools for biomedical research and therapy, in particular the anticancer strategy gene-directed enzyme prodrug therapy, and targeted cellular ablation in zebrafish models of degenerative disease. We have used directed evolution to improve desirable activities and are also investigating the use of dual positive and negative selection strategies to tailor reaction specificity and to better understand how the evolution of promiscuous enzymes is modulated by *in vivo* constraints. A further application of our positive selection capabilities has been the recovery of novel nitroreductases from collections of uncharacterised environmental DNA.

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Facile interconversion of a FMN-dependent dehalogenase and nitroreductase

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Keywords: dehalogenase, nitroreductase, flavin semiquinone, 5-deazaFMN

The flavoprotein iodotyrosine deiodinase (IYD) is very unusual for its ability to promote reductive dehalogenation under aerobic conditions and represents an unique function within its nitro-FMN reductase superfamily.1,2 Research on this enzyme has been motivated in part by questions on how disparate catalytic activities arise from common structural architectures. Interest is currently focused on how IYD adapts and controls the chemistry of its FMN to support catalysis.

Interconversion of IYD and nitroreductase was found to be surprisingly simple. These distinct activities are regulated by differential access to the one- and two-electron chemistry of FMN, respectively. A single amino acid substitution that affects hydrogen bonding to the N5 position of FMN is sufficient to switch between the seemingly unrelated ability to promote dehalogenation and nitro reduction.3 Thus, evolution in this example could have been driven by the mere replacement of one key amino acid. For native IYD, substrate binding is also critical for switching between one- and two-electron processes. Additionally, a FMN analog such as 5-deazaFMN can be used to enforce an obligate two electron transfer for stimulating nitroreductase activity and suppressing dehalogenase activity.4 IYD•5deazaFMN offers a rare opportunity to reduce a nitroaromatic substrate to its fully reduced amino product and avoid accumulation of the most common hydroxylamine intermediate. Reconstitution of IYD with 1-deazaFMN retains the reductive dehalogenase activity of the native enzyme as expected for a FMN analog that promotes one electron transfer processes.4

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Real-time analysis of conformational control in electron transfer reactions of diflavin oxidoreductases.

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Keywords: Electron transfer, FRET, Protein dynamics, diflavin oxidoreductase

Biological electron transfer and protein domain dynamics are often associated. However, as it is experimentally challenging to trap catalytically relevant conformational sub-states, little is known about the role of protein domain dynamics during enzyme catalysis. For the diflavin oxidoreductases family, the importance of dynamics has been inferred from a variety of structural, single-molecule, and ensemble spectroscopic approaches. However, no experimental methods have been able correlate diflavin oxidoreductase motions with chemical steps during turnover. To address this shortcoming, we have labelled two physiologically important diflavin oxidoreductases, cytochrome P450 reductase and neuronal nitric oxide synthase (nNOS), with donor and acceptor fluorophores. By using both UV-Vis and FRET stopped-flow approaches, we have been able to detect transient chemical and conformational states that occur during diflavin oxidoreductase catalysis. Moreover, by making use of 5-deazaflavin mononucleotide (as a thermodynamic block) and isotopically labelled nicotinamide coenzymes, we have correlated the timing of diflavin oxidoreductases structural changes with key mechanistic steps (coenzyme binding/reaction chemistry).

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High resolution studies of hydride transfer in the ferredoxin-NADP+ reductase superfamily

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Keywords: hydride transfer, crystallography, active site compression, NADPH oxidase

The flavoenzyme ferredoxin-NADP+ reductase (FNR) catalyzes the transfer of electrons from photoreduced ferredoxin to NADP+ during photosynthesis and serves as a model for a broad superfamily of enzymes including NO synthase, cytochrome P450 reductase, and NADPH oxidases. Our goal is to define the mechanistic details of hydride transfer between FAD and NADPH using corn root FNR as a model.

In initial studies of spinach FNR crystals, the nicotinamide binding site was seen to be blocked by an aromatic side chain (Tyr) lying close and parallel to the *re*-face of the flavin. Using a Tyr to Ser mutant of pea FNR, 1.8 Å resolution structures with NADP(H) bound were obtained,[1] revealing an unexpected binding mode in which the nicotinamide ring laid against the FAD isoalloxazine at a ~30° angle. Although similar complexes have been seen in other FNR superfamily members, based on stopped-flow studies it has been claimed that these complexes are non-productive.[2] To resolve this question and better define the mechanistic details of hydride transfer, we carried out spectroscopic studies and determined higher resolution structures of FNR-NADP(H) complexes.

Here, we present the structures of wild type corn root FNR at ~1 Å resolution, along with ~1.5 Å resolution Y316A and Y316S mutants in complex with nicotinamide, NADP+, and NADPH. These enzymes are active and spectra of the crystalline complexes match those from solution studies. Also a reinterpretation of the earlier stopped-flow studies supports the relevance of these complexes to catalysis. Furthermore, our structures reveal more detailed information about the hydride transfer reaction. In particular, the complexes show higher anisotropic mobility of the C4 atom of NADP+ compared to NADPH, very short contact distances between NADPH and FAD, and distortion of FAD geometry that implicate active site compression as a key factor enhancing hydride transfer in the FNR superfamily.

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Formate oxidase (FOX) activity is governed by autocatalytic formation of 8-formyl FAD exhibiting unusual properties

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Keywords: 8-formyl FAD, formate oxidase, glucose methanol choline, novel cofactor.

Formate oxidase (FOX; E.C. 1.2.3.1) from *Aspergillus oryzae* has been identified as the first and only member of the glucose-methanol-choline (GMC) oxidoreductase superfamily of enzymes to oxidize a carbonic acid. Additionally, wild-type FOX has been shown to exhibit an unusual UV absorption spectrum that was due to a non-covalently bound 8-formyl FAD in place of the typical FAD cofactor present in most GMC oxidoreductases. Although the presence of an enzyme bound 8-formyl FMN has been reported previously as a result of site-directed mutational studies on lactate oxidase (LOX), FOX is the first reported case of 8-formyl FAD being present in a wild-type enzyme. Since the formation of 8-formyl FMN in LOX has been shown previously to result in complete inactivation of the enzyme, the presence of 8- formyl FAD in FOX was proposed to be an artifact. Therefore, both the formation and role of the 8-formyl FAD cofactor in formate oxidase was investigated through the use of steady-state kinetics, rapid-reaction kinetics, kinetic isotope effects, site-directed mutagenesis, ICP analysis, UV and fluorescence spectrometry, LCMS, electron paramagnetic resonance (EPR) spectroscopy, analytical ultracentrifugation (AUC), and light-exposure studies. Surprisingly, the results from these studies not only indicate that 8-formyl- FAD is present in the active form of FOX but that its autocatalytic formation is crucial for activity. As a result, formate oxidase serves as the first enzyme reported to have an active 8-formyl FAD as a cofactor. The FOX bound 8-formyl FAD was also shown to form a highly stable anionic semiquinone when exposed to light.

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Flavin-dependent epitranscriptomic: when flavoenzymes regulate the translation

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Post-transcriptionally modified nucleosides in RNA play integral roles in the cellular control of biological information encoded by DNA. The chemical modifications of RNA span all three phylogenetic domains (Archaea, Bacteria, and Eukarya) and are pervasive across RNA types, including messenger RNA (mRNA), transfer RNA (tRNA), ribosomal RNA (rRNA), small nuclear RNA (snRNA) and microRNA (miRNA). Nucleotide modifications are also one of the most evolutionarily conserved properties of RNAs, and the sites of modification are under strong selective pressure. Among the RNA species, tRNAs are the most heavily modified. Approximately 15%–25% of all nucleosides in eukaryotic tRNA contain modifications. Although not fully understood, these modifications have been proposed to serve various purposes: (1) tRNA discrimination (e.g., initiator tRNAMet is distinguished from elongator tRNAMet through ribosylation at A64); (2) translation fidelity, where absence of modifications at wobble position 34 causes decoding errors because the modified base either restrict or extends codon-anticodon interaction capability through base pairing; and (3) tRNA stability and quality control of the macromolecule. To date more than a hundred different types of modifications have been reported and more will certainly be discovered in the future. The chemical diversity of these “new nucleotides” is astonishing and puzzling since no other biological macromolecule is subjected to such enormous biochemical transformation. Recently several flavoenzymes have been shown to participate in such RNA modifications using sophisticated mechanisms and unprecedented chemistry. The lecture will review our recent work on these flavin-dependent RNA modification enzymes.

Electron transfer flavoprotein goes moonlighting

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Keywords: Electron Transfer Flavoprotein, Fatty acid oxidation, LYRM5, mitochondrial respiratory complex I.

Mammalian electron transfer flavoproteins (ETF) are heterodimers containing a single FAD, and are located in the mitochondrial matrix. ETF serves as the electron acceptor for nine primary flavoprotein dehydrogenases involved in mitochondrial fatty acid and amino acid catabolism and for two enzymes in choline metabolism. Reduced ETF is re-oxidized by the membrane-bound ETF-ubiquinone oxidoreductase in an electron-transfer pathway linking the primary dehydrogenases with respiratory Complex III. In addition to its role in redox chemistry, ETF now appears to be involved in respiratory Complex I assembly via LYRM5. LYRM5 is a member of the LYRM protein superfamily (LYRMs). LYRMs are basic, approximately 15 kDa polypeptides and contain a conserved tripeptide L-Y-R (leucine/tyrosine/arginine) sequence near the N-terminus. The human genome contains at least ten LYRMs that were predominantly identified as mitochondrial proteins. Human LYRMs are linked with diseases, such as insulin-resistance (LYRM1), muscular hypotonia (LYRM3), deficiency of multiple OXPHOS complexes (LYRM4), to list a few. LYRM5 was shown to be a Complex I phylogenetic profile (COPP) protein by mitochondrial proteomics studies[1].

Recently, mitochondrial protein-interaction-mapping studies identified that LYRM5 strongly interacts with both subunits of ETF[2]. We have performed further biochemical studies using purified proteins of human ETF and LYRM5. The two proteins form a stable complex with a stoichiometry of ETF/LYRM5=1/4. Furthermore, LYRM5 efficiently releases the enzyme-bound FAD upon incubation with ETF, thereby inactivating the enzymatic activity of ETF. Limited trypsin digestion studies and co-expression of LYRM5 with various combinations of ETF domains suggest that LYRM5 binds to ETF at the concave surface of ETF, which is located at the cleft between the two subunits, and overlaps with the FAD binding site. Studies are in progress to determine the exact nature of interactions between ETF and LYRM5, and the non-oxidative role of ETF in Complex I assembly.

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Human MICAL-1: the multidomain flavoenzyme participating to actin cytoskeleton dynamics

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Keywords: MICAL, oxidase/monooxygenase, F-actin depolymerization, oxidative stress.

MICAL (from the Molecule interacting with CasL) indicates a family of multidomain proteins conserved from insects to humans, which are increasingly attracting attention for their participation in the control of actin cytoskeleton dynamics, and, therefore, in the several related key processes in health and disease. MICAL is unique among actin binding proteins because it catalyzes a NADPH-dependent F-actin depolymerizing reaction. This unprecedented reaction is associated with its N-terminal FAD-containing domain that is structurally related to p-hydroxybenzoate hydroxylase, the prototype of aromatic monooxygenases, but catalyzes a strong NADPH oxidase activity in the free state. The additional calponin homology (CH), LIM (from Lin-11, Isl-1 and Mec-3 gene products) and C-terminal regions, which are typical protein interaction domain, are believed to play modulatory roles. In order to contribute to the understanding of this class of important enzymes we have produced full-length human MICAL-1 and truncated forms progressively lacking the C-terminal region, the LIM and also the CH domain1-3. From their comparison it is emerging that the MICAL-1 NADPH oxidase activity may contribute to its biological role and it is also responsible of the F-actin depolymerizing activity, as opposed to the hypothesis that MICAL switches from an oxidase (in the free state) to a specific actin methionine hydroxylase (when F-actin bound). The CH and LIM domains increase the K_m for NADPH of the free enzyme but have no effect on the reaction in the presence of F-actin. The C-terminal region is responsible of the MICAL-1 autoinhibition, which is part of the biological control mechanism. The equilibrium between the active and active MICAL conformations in solution is being studied by combining kinetics and structural approaches also using MICAL interactors.

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Photoactivation mechanisms of flavin-binding photoreceptors

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Flavin-binding photoreceptor proteins regulate a wide variety of light responses in plants, algae, bacteria and fungi, and have recently gained attention as important optogenetic actuators. Here, I will present photoactivation mechanisms of LOV and BLUF domains assessed through the application of time-resolved optical and vibrational spectroscopy, encompassing the photon absorption event on the femtosecond timescale, the photochemistry of the flavin and amino acid side chains in picoseconds to microseconds, all the way through protein secondary structural changes on the microsecond to millisecond timescales.

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Light and redox sensing by flavoproteins

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Keywords: cryptochromes, LOV-domain proteins, transmembrane signaling, photoreduction

Blue-light sensing proteins coordinate many biological processes that include phototropism, photomorphism, stress responses, virulence and the entrainment of circadian clocks. Three major types of blue-light sensors all bind flavin nucleotides as chromophores, but the photochemistry employed and conformational responses invoked differ considerably among the classes. Nevertheless, photoinduced electron transfer reactions play a key role in several mechanisms. How such reactivity leads to conformational signaling will be discussed for both cryptochromes (CRYs) and light- oxygen- voltage (LOV) domains. In CRYs, blue-light mediated flavin reduction promotes proton transfer within the active center that then leads to displacement of a key signaling element. For LOV proteins, blue light causes formation of a covalent cysteinyl-flavin adduct, which rearranges hydrogen bonding and restructures the N-terminal region of the protein. Interestingly, a new class of LOV-like sensor does not undergo adduct formation and instead can operate by flavin photoreduction, like CRY. In yet another related system, flavin reduction of a LOV-like domain serves to regulate transmembrane signaling. Conserved aspects of reactivity in these proteins provide lessons for the design of new photosensors, which may find use as tools in optogenetics.

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Use of *R*-amine oxidase evolved from D-amino acid oxidase for chiral (*S*)-amine synthesis and oxidative cyanation reaction

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Keywords: *R*-amine oxidase. D-amino acid oxidase. deracemization. oxidative cyanation

We will report our recent results on the generation of a new enantiocomplementary [1] *R*-stereoselective amine oxidase, by protein engineering from porcine kidney D-amino acid oxidase (pkDAO) [2]. The new enzyme was evolved based on our mechanism- and structure-based speculation, and screening on (i) intermediate imine formation of oxidoreductases acting on amines, (ii) highly similar tertiary structures between flavin-dependent *S*-amine oxidase (*Aspergillus niger*) and L-amino acid oxidase (snake venom), and (iii) the relationship as enantiocomplementary enzymes between L-amino acid oxidase and pkDAO.

pkDAO catalyzes the oxidative deamination of D-α-amino acids with strict *R*-stereoselectivity, but it does not act on simple amines. Engineered pkDAO Y228L/R283G with markedly changed substrate specificity toward (*R*)-amines was obtained. The mutant enzyme exhibited a high preference toward the substrate (*R*)-α-methylbenzylamine, totally losing its original activity toward D-amino acids. The crystal structure of the mutant enzyme revealed the binding of (*R*)-α-methylbenzylamine to the active site. The mutant enzyme was used to synthesize (*S*)-amine through deracemization from racemic α-methylbenzylamine by a selective oxidation of (*R*)-amine in the presence of a chemical reductant such as $NaBH_4$. We also have been successful to engineer the enzyme to accommodate amines with bulkier substituents.

Oxidation of amino groups in amines or amino acids activates the sp^3 Cα-H bond to form imines, making the alpha carbon atom a target for nucleophile reagents such as cyanide. Mutant pkDAO (Y228L/R283G) catalyzed the synthesis of racemic-2-methyl-2-phenylglycinonitrile from (*R*)-α-methylbenzylamine and KCN. Based on these results, we developed a new cascade reaction for the synthesis of unnatural α-amino acids from primary amines using mutant pkDAO and nitrilase AY487533 [3].

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Biotechnological application of human FMO3: an electrochemical biochip for drug metabolism

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Keywords: Flavin-containing monooxygenase, immobilisation, graphene, LC-MS.

Human hepatic Phase I drug metabolising enzymes include the large family of cytochromes P450 and flavin-containing monooxygenases. In general, the activity of these enzymes renders the drugs and xenobiotics more amenable to excretion. Human flavin-containing monooxygenase 3 (hFMO3) is a microsomal enzyme containing a single non-covalently bound FAD as its cofactor. This enzyme is NADPH-dependent and is able to use molecular oxygen to catalyse the oxygenation of a variety of structurally different xenobiotics, including many therapeutic drugs.

The presence of its flavin cofactor makes it suitable for electrochemical investigations, even though this enzyme similar to other membrane-bound enzymes, is difficult to manipulate in *in vitro* setting for biotechnological applications. Our group has been involved in studying the wiring of this human enzyme to electrode surfaces such as gold and carbon for more than a decade. Electrode modification for immobilisation of this enzyme has been achieved in a number of ways, ranging from the use of films of positively charged surfactants such as didodecylammonium bromide (DDAB), poly-(dimethyldiallylammonium chloride) (PDDA) to ordered self-assembled thiol-terminated chains such as cystamine-maleimide. More recently, we have also demonstrated the successful use of gold nanoparticles (AuNps) and graphene[1]. Data will be presented regarding the activity as well as identification of the metabolite(s) produced by this human enzyme and several of its polymorphic variants[2] once immobilised on electrode surfaces. The successful development of this type of biochip has direct applications in the era of personalised medicine.

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Enzymatic redox-neutral convergent cascade for lactonizations

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Keywords: Enzymatic cascades, Baeyer-Villiger monooxygenase, cofactor regeneration, cofactor specificity

Nature uses an extraordinary synthetic strategy by building multi-step biotransformations *via* coupling of enzymes.1 The elegance and efficiency of natural cascades and networks also explains the increasing popularity of domino- or cascade reactions in organic synthesis in general. Especially in redox biocatalysis, cascade reactions are attractive since with the so-called redox-neutral cascades expensive cofactors can be recycled *in situ* without an additional cofactor regeneration system.2

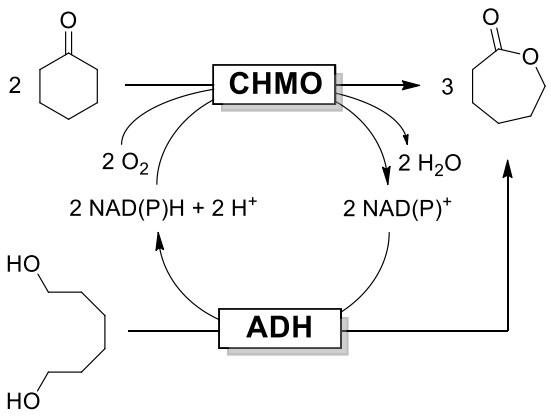

Figure 1. Redox-neutral convergent cascade coupling a BVMO and an ADH.

Recently, we reported a new concept of redox-neutral convergent cascade reaction consisting of an alcohol dehydrogenase (ADH) and a cyclohexanone monooxygenase (CHMO) (Figure 1).[3] The cofactor regeneration of this cascade is achieved *via* oxidation of the "double-smart cosubstrate" 1,6-hexanediol catalyzed by the ADH. Two molar equiv. of cyclohexanone and one molar equiv. of 1,6-hexanediol can be converted into a single product ε-caprolactone with high atom efficiency. Productivity was optimized with respect to the process parameters *via* a two-step Design of Experiments approach and a biphasic system.[4]

The low process stability and product inhibition issues observed with the CHMO from *Acinetobacter sp.* NCIMB 9871 ($CHMO_{Acineto}$) motivated us to evaluate new CHMO variants or novel Baeyer-Villiger monooxygenases (BVMOs). Two newly designed CHMO variants (CHMO M15 L323C-A325C and CHMO M16 L323C-A325C) and a new BVMO from *Aspergillus flavus* NRRL3357 (AFL706) were characterized in terms of enzyme kinetics and stability. High long-term stability and reduced product inhibition of these enzymes allow high potential for biocatalytic applications.5 The above mentioned convergent cascade has been limited to NADPH due to the strict cofactor recognition of $CHMO_{Acineto}$. A recently discovered type II flavin-containing monooxygenase (FMO-E) from *Rhodococcus jostii* RHA1 with relaxed cofactor specificity inspired us to evaluate its application for the synthesis of lactones. Up to 90% analytical yields could be detected when combining FMO-E with horse liver ADH in a NADH-dependent convergent cascade fashion.6

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On the evolution of flavin-dependent enzymes: retracing the steps

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The limited number of protein domains and cofactors that Nature has employed to grow an astounding variety of enzymatic functions, will never cease delighting us. Although enzymes are highly flexible molecules, they form thermodynamically stable structures and therefore their evolutionary trajectories are constrained to a narrow range of pathways. Understanding the evolutionary history of an enzyme family will reveal the complex network of changes that have occurred from ancestral sequences leading to the emergence of extant enzymes. This will allow us to better comprehend enzyme mechanisms and identify the essential subset of residues responsible of functional differences between family members. Moreover, dynamics of enzyme evolution can unveil the functional constraints and contingencies on how a new enzyme function arises, and ultimately what makes an enzyme to display a certain activity.

In this context, the evolutionary history of two different enzyme families (meaning, a set of enzymes displaying the same fold and active site features and thus likely to share a common ancestor) was studied. Flavin-dependent monooxygenases are a functionally diverse family, including aromatic hydroxylases, Baeyer-Villiger monooxygenases and epoxidases. By integrating data from sequences, 3D structures, multidomain architectures, chemistry and phylogenetic inferences, we were able to unveil a complex set of factors influencing the evolution of this family and leading to its current diversity[1,2]. By a different approach, we have focused on resolving the evolutionary relationships of enzymes using F_{420} and/or FMN as cofactor. Particularly, how reductases and dehydrogenases, with a focus on FGDs (glucose-6-phosphate dehydrogenases)[3], emerged from an FMN-dependent ancestor and diverged into the current functional variety, has been addressed. Moreover, by using the ancestral sequence reconstruction approach and resurrecting enzymes in the lab, the emergence of the dehydrogenase function has been traced.

These examples show how phylogeny and bioinformatics bridge the gap between biochemistry and molecular evolution and provide insight on how a new enzyme-function emerges. This offer valuable information for enzymology related research and may facilitate more effective enzyme discovering and enzyme engineering efforts.

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Structure, function and evolution of $F_{420}H_2$ utilizing flavin/deazaflavin oxidoreductases (FDORs) in Mycobacteria

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Keywords: deazaflavin(F_{420}), mycobacteria, oxidoreductases, biliverdin reductase

Mycobacteria produce a rare deazaflavin cofactor F_{420} found in Actinobacteria and Archaea, which is important for persistence during oxidative stress, including in pathogenic species like *Mycobacterium tuberculosis*. However, the mechanisms of F_{420} utilization in mycobacteria remain largely unknown. We have characterized the largest mycobacterial F_{420} utilizing protein family called the flavin/deazaflavin dependent oxidoreductases (FDORs), to identify their functional roles in aiding mycobacterial infection and persistence. This family also consists of proteins with cofactor specificities for FMN, FAD and heme. By solving five novel X-ray crystal structures of FDORs and comparing them with previously available structures, we showed that FDORs have relatively conserved cofactor binding regions and extensively modified substrate binding pockets for variable functions.

Their cofactor preference, sequence similarity and existing characterizations allowed classification into functional groups, including the $F_{420}H_2$-dependent quinone reductases that also activate 4-nitroimidazole pro-drugs approved for combating multi-drug resistant *M. tuberculosis*. Novel FAD binding proteins that could be involved in triggering mycobacterial dormancy were also discovered, and *in silico* substrate docking was used to identify $F_{420}H_2$-dependent fatty acid saturases and $F_{420}H_2$-dependent biliverdin reductases (F-BVRs). Characterization of the F-BVR Rv2074 from *M. tuberculosis* showed its ability to reduce biliverdin-IXα to bilirubin-IXα, which is a potent antioxidant that could aid *M. tuberculosis* survive oxidative stress encountered inside macrophages during infection. This $F_{420}H_2$-dependent biliverdin reduction by Rv2074 is mechanistically similar to the NAD(P)H-dependent reactions in the mammalian biliverdin reductases as inferred using the structure of the Rv2074:F_{420} complex with biliverdin modelled into the active site.

Lastly, we showed that F_{420} production is more widespread in the bacterial community than previously thought, where Proteobacteria and Chloroflexi also produce F_{420} and encode FDORs for its utilization. Overall, this body of work characterized novel flavin/deazaflavin proteins, aiding to uncover the role of the rare deazaflavin cofactor F_{420} in bacterial metabolism. 2

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Trimming proline dehydrogenase: protein and cofactor minimization

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Proline dehydrogenase (ProDH) catalyzes the FAD-dependent oxidation of proline to Δ^1-pyrroline-5-carboxylate, the first step of proline catabolism in many organisms. Next to being involved in a number of pathological processes, ProDH is of interest for the enzymatic synthesis of peptides and antibiotics. ProDH is a membrane-associated aggregation-prone protein. We researched proline dehydrogenase from *Thermus thermophilus* (*Tt*ProDH) in order to gain more insight into the structure-function relationship of this thermo-resistant flavoenzyme.

*Tt*ProDH contains, next to a distorted TIM-barrel, three N-terminal helices, αA, αB and αC, of which the function is not well understood. We constructed helical arm-truncated variants, lacking respectively one (ΔA), two (ΔAB), or three (ΔABC) helices. ΔA and ΔAB are highly active tetramers, whereas removal of the complete N-terminal arm (ΔABC) results in poorly active dimers. In agreement with these results, ΔA and ΔAB rapidly react with *N*-propargylglycine, while ΔABC is not capable of forming a flavin adduct with this suicide inhibitor. Additional point mutations in helix C showed that a hydrophobic patch between helices αC and α8 is crucial for substrate recognition and tetramer formation.

The quaternary structure of *Tt*ProDH was investigated in more detail by disrupting two ionic interactions at the dimeric interface. By doing so, monomers of *Tt*ProDH are formed, which have improved catalytic properties at moderate temperatures compared to their tetrameric counterparts. However, their melting temperatures are decreased by more than 20 °C, indicative for a trade-off between thermostability and catalytic activity.

We also studied the cofactor binding of *Tt*ProDH. For that, the enzyme was produced in its apoform using a riboflavin auxotrophic *E. coli* strain. Reconstitution of the enzyme with either FAD or FMN revealed that *Tt*ProDH has no preference for FAD as cofactor. Furthermore, the holo form of *Tt*ProDH, as produced in *E. coli* TOP10 cells, contains about three times more FMN than FAD. The capability of *Tt*ProDH to display equal properties with both cofactors is rather unusual among flavoenzymes.

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The structural prerequisites for enzymatic flavin-N5-oxide formation

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Keywords: flavin-N5-oxide, monooxygenase, X-ray crystallography, oxygen binding

Flavoenzymes play key roles in the generation of numerous chemically complex natural products, as exemplified by the FAD-dependent EncM of *Streptomyces maritimus*, which catalyzes an oxidative carbon-carbon rearrangement and triggers a cyclization cascade in the biosynthesis of the structurally unique polyketide antibiotic enterocin. We discovered that EncM employs a flavin-N5-oxide oxygenating species - rather than the commonly observed flavin-C4a-peroxide - for the oxidation of the polyketide substrate1,2. Currently, we investigate the structural features of EncM that promote formation of this novel flavin redox state via mass spectrometry and protein crystallography among other techniques. We propose that flavin-N5-oxide formation in EncM depends on a highly defined oxygen-binding pocket consisting of a few mostly hydrophobic amino acid residues in close proximity to N5 of the isoalloxazine ring. The reaction of reduced flavin with oxygen appears to be rigorously controlled by the protein microenvironment, as minor modifications of this pocket via site-directed mutagenesis were sufficient to preclude flavin-N5-oxide formation in the EncM variants. Oxygen binding in this pocket was furthermore corroborated using computational approaches as well as X-ray crystallography. For this purpose, EncM crystals where mounted in a pressurizable cell, exposed to various oxygen overpressures (5, 10, 15 bars, respectively), flash frozen and directly measured. Compared to EncM crystals prepared under atmospheric oxygen partial pressure (0.21 bar), a clear increase in electron density was observed in the proposed pocket consistent with an increased occupancy of oxygen. These results contribute to our understanding of how flavoproteins control their reaction with O_2 and may facilitate rational approaches to redesigning and engineering flavin oxygenases and oxidases.

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Cryptochromes and perception of electromagnetic fields: role of the redox photocycle and potential signaling mechanisms

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Keywords: cryptochromes, photoreduction, Reactive Oxygen, magnetosensing

Cryptochromes are wide-spread evolutionarily conserved flavoprotein receptors with diverse sensory functions in organisms ranging from plants to humans. Cryptochromes are activated by a photocycle involving flavin photoreduction (1) and the biosynthesis of ROS (2,3). Though initially characterized as light sensors, cryptochromes have also been proposed as possible magnetosensors based on photochemical properties which are compatible with the Radical Pair Model of chemical magnetosensing (4). This suggestion is supported by increasing evidence of cryptochromes implicated in magnetosensing in multiple organisms.

In this presentation we show data using isolated proteins on the conserved photochemical characteristics of the cryptochrome photocycle that are compatible with signaling and radical pair formation. We then probe magnetic sensitivity *in vivo* under defined light and magnetic field conditions using the drosophila, plant, and migratory bird model systems to identify the most likely magnetosensitive step in the cryptochrome photocycle. Our results exclude the currently held hypothesis that magnetosensing by cryptochromes could occur in the course of light-induced forward electron transfer through the conserved Trp triad to the flavin radical, as proposed in numerous studies to date (5). Instead, magnetosensing occurs during the light-independent flavin reoxidation step, with some form of reactive oxygen species (ROS) as a likely intermediate.

Finally, we provide evidence for the first time that cryptochromes are involved in magnetic sensitivity to weak (low mT range) magnetic fields in mammalian and human cells, and that this cryptochrome magnetosensing mechanism also does not require light and involves the formation of ROS.

These results will be discussed with respect to the prevailing models of magnetoreception currently in the literature, as well as with respect to disturbing implications on the possible long-term effects of exposure to electromagnetic noise in the environment may have on public health.

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Enantioselectivtiy of styrene monooxygenases towards sulfides is defined by a tyrosine in the active site

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Keywords: styrene monooyxgenase, enantioselectivity, sulfoxidation, phylogeny.

Styrene monooxygenases (SMOs) are flavoproteins consisting of two-components, the monooxygenase part (StyA) and the reductase part (StyB) [1]. The latter one delivers reduced FAD to StyA, which is used to activate molecular oxygen for the reaction with an aromatic substrate. SMOs can be distinguished into E1-type enzymes, which are usually part of a styrene degradation cluster and an E2-type, that is not part of a certain cluster, but supposed to be involved in the detoxification of indole. The E2-type includes an oxygenase StyA1 and a construct StyA2B representing a natural fusion of StyA and StyB into a single protein [2]. SMOs catalyze the regio- and enantioselective epoxidation of styrene to (*S*)-styrene oxide but also indole oxygenation and selective sulfoxidations, which are of interest for industrial application. However, the preference for an enantiomer can differ.

To rationalize this finding, a phylogenetic analysis of known and putative SMOs was conducted. 25 candidates from different branches were selected, cloned, expressed and screened for indigo formation. 7 of them showed no color formation in this test, indicating inactivity towards indole and/or malfolding. The remaining 18 SMOs were synthesized, purified and characterized according to activity, substrate spectrum and enantioselectivity.

Results obtained clearly show that the activity differs randomly among the groups. Interestingly, in most cases the sulfoxidation activity towards thioanisole and benzyl methyl sulfide is higher compared to styrene. Further, there is a clear prevalence towards the (*R*)-enantiomer for the E1-type and the (*S*)-enantiomer for E2-type SMOs in sulfide conversions. Lin et al. showed by mutation that a tyrosine in the active site changes the enantioselectivity for 1-phenylcyclohexanone [3]. Sequence analysis as well as structural modelling indicate that this amino acid change also reflects the enantioselectivity towards sulfides of E1- and E2-type SMOs and that Tyr73 acts as the guiding residue for substrate binding.

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Playing on both sides of the mirror:amino acid oxidases as versatile tools for chiral biocatalysis

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Keywords: D-amino acid oxidase, L-amino acid oxidase, L-amino acid deaminase, deracemization, chiral biosynthesis, biocatalysis.

The increasing demand for enantiomerically pure compounds from the chemical, pharmaceutical and agrochemical industry renewed the interest on the otherwise "old" enzymes D-amino acid oxidase (DAAO) and L-amino acid oxidase (LAAO) whose existence was demonstrated in the first half of the past century. Enantioselective biocatalysis, a competitive approach in comparison to classical asymmetric synthesis, requires strict enantioselective enzymes. DAAO turned out to be a versatile biocatalyst and a robust scaffold to evolve novel variants active even on unnatural amino acids. For this reason, this enzyme was exploited to produce biosensors, for the synthesis of pure L-amino acids, and even for enzyme therapy[1]. On the other hand, biotechnological exploitation of LAAOs turned out to be an awkward task because of the difficulty in their recombinant overexpression. On this side, the recent characterization of an L-amino acid deaminase from *Proteus myxofaciens* (PmaLAAD) allowed to close the circle and to extend the application of chiral biocatalysis also to the production of pure D-amino acids[2].

PmaLAAD is a member of a novel family of flavoproteins associated to the cellular membrane that catalyze the oxidative deamination of L-amino acids without production of H_2O_2. Electrons are transferred from the reduced cofactor of the enzyme to a cytochrome b-like protein. PmaLAAD is specific for large hydrophobic L-amino acids (e.g., L-Phe) and for several natural (e.g., L-DOPA) or unnatural (e.g., L-1-naphthylglycine, substituted alanines) L-amino acid derivatives. Although the overall 3D structure of the enzyme is similar to the one of known amino acid oxidases, PmaLAAD shows peculiar features, such as a transmembrane α-helix, an unusual additional α+β subdomain that interacts with the membrane and a large, accessible and partially flexible active site[2].

The wild-type PmaLAAD and its evolved variants turned out to be competitive and versatile biocatalysts for deracemization and stereoinversion of amino acids of industrial interest.

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A light-driven cascade to promote peroxygenase catalyzed reactions

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Keywords: biocatalysis, photochemistry, regeneration system, oxidation

The oxyfunctionalization of non-activated C-H bonds is one of the greatest challenges in organic chemistry [1,2]. Peroxygenases are promising catalysts that can achieve selective hydroxylation of non-activated C-H bonds using hydrogen peroxide (H_2O_2) as oxidant. However, these enzymes exhibit poor robustness against the latter. Therefore, methods for the in situ supply with H_2O_2 have to be investigated. Most commonly, this is achieved by catalytic reduction of molecular oxygen [3,4].

One promising approach may be to use formate as a sacrificial electron donor to promote the reductive activation of O_2. Here, we propose a photochemoenzymatic method comprising formate dehydrogenase and flavins under visible light irradiation (Figure 1). In this approach, H_2O_2 is produced by the spontaneous reduction of O_2 by a reduced flavin (FMN_{red}). This flavin (FMN_{ox}) is then regenerated by an photochemoenzymatic system. The photoexcitation of FMN_{ox} by visible light enables its reduction by NADH which is regenerated by the consumption of formate by formate dehydrogenase (FDH).

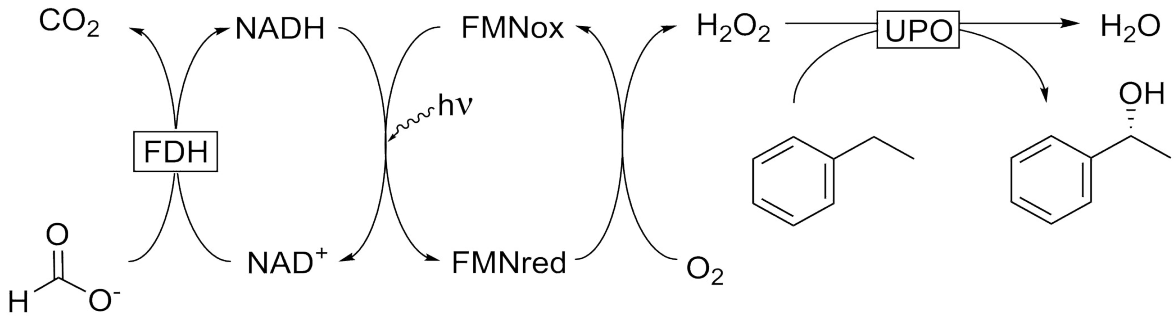

Figure 1. Photochemoenzymatic generation of H_2O_2 to promote Unspecified Peroxygenase (UPO) catalyzed hydroxylation.

The setup and characterization of this novel approach will be discussed.

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Berberine bridge enzyme-like proteins: from characterization to application

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Keywords: Berberine bridge enzyme-like proteins, monolignol oxidoreductase, vanillyl-alcohol oxidase flavoprotein family, lignin

Flavoproteins are a diverse protein class employing an isoalloxazine ring for catalysis in form of the flavin mononucleotide (FMN) or the flavin adenine dinucleotide (FAD). Among them, is the berberine bridge enzyme-like (BBE-like) protein family (pfam 08031) that was named after the berberine bridge enzyme (*Ec*BBE) from California poppy (*Eschscholzia californica*). BBE-like proteins form a multigene family in plants and the number of members varies from one in the moss *Physcomitrella patens* to 28 in Arabidopsis thaliana and 57 in the Western Balsam Poplar (*Populus trichocarpa*). Despite of the frequent occurrence of these proteins their function is largely unknown. Therefore, we chose to investigate the BBE-like proteins occurring in *A. thaliana* to broaden our understanding of this protein family. Presented will be the structural and biochemical characterization of *At*BBE-like protein 15 and 28, the application of an engineered *At*BBE-like 15 variant as biocatalyst, the role of *At*BBE-like 15 in plant physiology and the development of the BBE-like protein family in the plant kingdom.

Our analysis of the *At*BBE-like protein family reveals that there are four frequently occurring active site types in plants1,2. *At*BBE-like protein 15 is a representative of the most abundant type, therefore it can serve as a paradigm for the majority of these proteins. The enzyme was identified as monolignol dehydrogenase, an activity that was not recognized for this protein family before. Additionally, the enzyme was rationally engineered towards higher oxygen reactivity and the potential of the enzyme as biocatalyst for oxidative reactions was tested3. Experiments with *At*BBE-like 15 knock out plants revealed a perturbation of the content and the total amount of lignin in these plants. This indicates that monolignol dehydrogenases are responsible for the manipulation of the monolignol pool and represent an electron delivery system to fuel the lignification that was previously not recognized.

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Fungal flavoenzymes involved in cellulose degradation

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Keywords: cellobiose dehydrogenase, fungal oxidoreductases, cellulose degradation, lytic polysaccharide monooxygenase

Secreted, FAD-dependent oxidoreductases belonging to the glucose-methanol-choline (GMC) superfamily are ubiquitously distributed in wood decaying fungi. Their exact physiological roles often remain elusive, but have been frequently associated with fungal biomass degradation. Here, we analyzed the structure and function of the extracellular flavocytochrome cellobiose dehydrogenase (CDH, CAZy AA3.1), which is secreted during growth on cellulosic substrates. Using mutational analyses in combination with steady-state and pre-steady state techniques we showed that CDH catalyzes the flavin-dependent oxidation of soluble cellulosic and hemicellulosic sugars, while a flexible cytochrome domain transfers these reduction equivalents from the FAD to external interaction partners[1,2]. Our data support the proposed physiological function of CDH as a reductase for copper-dependent lytic polysaccharide monooxygenase (LPMO, CAZy AA9), which cleaves crystalline cellulose surfaces and boosts the performance of hydrolytic cellulases. A genomic survey showed a high overlap of *cdh* genes with *lpmo* genes in saprotrophic fungi (~70%), but indicated less co-occurrence of these genes in fungi with a limited number of cellulose-hydrolyzing enzymes. Our data also provide evidence that other secreted FAD-dependent enzymes, such as glucose dehydrogenase, could be connected to the degradation of cellulose[3]. These enzymes can activate LPMO indirectly by reduction of low-molecular mass plant phenols, which can serve as efficient redox mediators between LPMO and GMC oxidoreductases. Arguably, fungi secreting GMC oxidoreductases as reduction systems for LPMO can better adapt to changing environmental conditions. The fusion of one of these GMC oxidoreductases with a cytochrome domain evolved CDH as a specific and efficient electron transfer system. These findings may shed light on the abundance and multigenicity of GMC oxidoreductases in fungal genomes and could help understanding their roles in biomass degradation.

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Drug development for cancer treatment and prevention targeting the human NAD(P)H: quinone oxidoreductase 1

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Keywords: NQO1P187S, cancer, drug, chemotherapy

Human NAD(P)H quinone oxidoreductase 1 is one of the most extensively studied flavoproteins.1,2 Its enzymatic role in the antioxidative defense system as well as the role in stabilising various tumour suppressors and other labile proteins was studied in various laboratories.3,4,5 Due to an increased NQO1 concentration in several tumour cells the use of chemotherapeutics that are activated by NQO1 is already well established.6 In current studies the role of the C609T single nucleotide polymorphism, leading to a proline serine exchange (NQO1 P187S), is investigated in particular. This exchange leads to a reduced activity and stability of the enzyme.7 The influence of the widespread NQO1 P187S polymorphism for a higher risk of cancer development and the failure of different chemotherapies is currently the target of research.8,9

Our former paper7 already provided the structural explanation for the reduced stability and loss of function of NQO1 P187S. Furthermore, we proposed the use of a molecular chaperone to rescue the stability and activity.7 One focus of a possible drug compound, able to support chemotherapy and prevent cancer development, is an increased stability of the target protein. A higher stability will lead to an elevated NQO1 level in the cells and thereby increase the overall enzymatic activity as well as the overall regulatory activity of the tumour suppressors. Another focus of the drug development is the increase of enzymatic activity. Therefore, the use of enzyme inhibitors is not reasonable since the benefit of a higher stability will be undone by a further loss of enzymatic activity. Possible candidates were found with an enhanced *in silico* screening method. A molecular chaperone can already increase the thermal stability of NQO1 as well as the turnover rate significantly. Thus, we have demonstrated that molecular chaperones may be used for the stabilization of unstable proteins.

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Tuning the regioselectivity of a robust cyclohexanone monooxygenase by structure-inspired enzyme engineering

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Keywords: Baeyer-Villiger oxidation, biocatalysis, cyclohexanone monooxygenase, regioselectivity

Flavin-dependent monooxygenases catalyze the incorporation of one atom of dioxygen into an organic substrate, reducing the other oxygen atom to water. Their catalytic cycle generally involves a C4a-(hydro)peroxyflavin intermediate, which can perform very different chemical tasks, including hydroxylations, Baeyer-Villiger (BV) oxidations, sulfoxidations, epoxidations, halogenations, and decarboxylations.1 We are especially interested in BV reactions, which are currently one of the most important transformations in organic synthesis. BV monooxygenases (BVMO) are an environmentally friendly alternative to using percarboxylic acids for converting ketones into esters or cyclic ketones into lactones.2

As an example of the many industrially relevant reactions performed by BVMOs, we will present the conversion of 2-butanone catalyzed by cyclohexanone monooxygenase (EC 1.14.13.22) from *Acinetobacter calcoaceticus* (AcCHMO). This reaction gives the regioisomers ethyl acetate and methyl propanoate.3 Methyl propanoate is a precursor of acrylic plastic. To improve the performance of AcCHMO, various mutants have been prepared and analyzed. The best mutant, T56S/I491A, exhibited a significant increase in both the activity on 2-butanone and the methyl propanoate/total product ratio.4

Recently, we discovered the first robust CHMO in the genome of *Thermocrispum municipale* (TmCHMO).5 TmCHMO presents a Ser residue at the equivalent position to that of AcCHMO T56. The TmCHMO I493, equivalent to AcCHMO I491, was replaced with an Ala by site-directed mutagenesis. The resulting mutant exhibited a similar activity and regioselectivity to those observed for T56S/I491A AcCHMO using the substrate 2-butanone. This work shows that it is possible to tune the regioselectivity even for a relatively small aliphatic substrate by structure-inspired enzyme engineering. Since TmCHMO is significantly more thermostable and solvent tolerant than all CHMOs described so far, and we could determine its crystal structure, it is a promising target for the generation of mutants relevant to biotechnology.

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The ins and outs of vanillyl alcohol oxidase: identification of ligand migration paths

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Keywords: ligand diffusion, flavoprotein, oxygen reactivity, Protein Energy Landscape Exploration (PELE)

Vanillyl alcohol oxidase (VAO) from *Penicillium simplicissimum* is a homooctameric flavoenzyme with a covalently bound FAD cofactor. The VAO-dimer is the smallest functional unit, and each subunit contains two domains, the FAD-binding and the cap-domain. Amongst the many reactions VAO catalyses is the two-step conversion of p-creosol (4-methylphenol) to vanillin (4-hydroxy-3-methoxybenzaldehyde).

We have performed Monte Carlo based simulations on dimeric VAO to understand how the substrates and products of the abovementioned reaction enter and exit the active site. For the phenolic ligands, one entry/exit path via the subunit interface leading to the *si*-side of the flavin, as well as two additional exit paths were identified. For the co-ligands dioxygen and hydrogen peroxide, a fourth path at the interface of the two domains of the VAO subunit, leading to the *re*-side of flavin, was discovered.

We propose a sequence of events leading substrates to the active site and products away from the active site. We also propose two concierge residues, which grant access to the active site for phenolic ligands as well as two gateway residues involved in co-ligand migration to the active site.

This study presents for the first time a comprehensive overview of ligand and co-ligand migration in a member of the VAO/PCMH family.

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Restricting FMN domain freedom and its impact on electron transfer and catalysis in the NO synthase flavoprotein

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Keywords: protein dynamics, kinetics, cross linking, hemeprotein

The nitric oxide synthase (NOS) flavoprotein domain contains linked NADPH/FAD- and FMN-binding domains. During catalysis, NADPH electrons transfer into FAD and into FMN for ultimate transfer to internal or external heme groups. Conformational switching of the FMN domain between an NADPH/FAD-associated (closed) and more free states is thought to be key for catalysis of electron transfer (ET) reactions, and FMN domain freedom may be controlled by regulatory elements in NOS and by calmodulin (CaM) binding. To address this, we are utilizing a "Cys-lite" NOS flavoprotein domain[1] and incorporating Cys pairs at interface junctions between the FMN domain and its domain or regulatory element partners, in order to restrict its conformational freedom via crosslinking. For example, we have substituted Cys at two residues that form a salt bridge between the NADPH/FAD and FMN domains in the closed structure, to allow domain-domain crosslinking by disulfide bond or by bis-maleimides of various length. The disulfide crosslink caused a ≥95% loss of cytochrome *c* reductase activity and was DTT-reversible. Graded crosslink lengthening gradually increased activity, thus helping define the conformational constraints on the FMN domain in this catalytic process. We used spectroscopic and stopped-flow techniques to investigate how changing the FMN domain conformational freedom impacts (i) the NADPH interaction, (ii) flavin reduction kinetics, (iii) stabilization of open versus closed conformational forms in two different flavin redox states, (iv) reactivity of the reduced FMN toward cytochrome *c*, (v) response to CaM, and (vi) the rates of interflavin ET and the FMN domain conformational dynamics. We are applying this approach to test how NOS regulatory elements control FMN freedom and function. Together, our findings reveal how spatial behaviors of the FMN domain impact catalysis, and how they are governed to control electron flow through the enzyme.

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Unraveling the role of riboflavin and its derived cofactors in health and diseases

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Keywords: Riboflavin, FAD synthase, neuromuscular disorders

The primary role of the water-soluble vitamin B2, i.e. riboflavin, in cell biology is connected with its conversion into FMN and FAD, the cofactors of a large number of flavoenzymes involved in energetic metabolism, redox homeostasis, protein folding, as well as in diverse regulatory events. Deficiency of riboflavin in humans has been linked to several diseases, including cancer and neuromuscular and neurological disorders, such as the Brown-Vialetto Van Laere syndrome [1]. In this contest, riboflavin at pharmacological doses has been shown to play unexpected and incompletely understood regulatory roles [1].

Besides a brief survey on riboflavin-related diseases and a summary on the state of art on riboflavin uptake mechanisms, remaining challenges in molecular characterization and sub-cellular compartmentation of FAD synthase (FADS), i.e. FMN:ATP adenylyl transferase (EC 2.7.7.2) encoded in humans by FLAD1 gene, will be presented [2]. FADS functional integration with other components of the cellular riboflavin/FAD network will also be depicted, as studied in different cellular and organism models [3].

Finally, experiments aimed at demonstrating FADS involvement as a component of the machinery that delivers FAD to apo-flavoproteins will be discussed, in the frame of the recent discovery of the link between alterations of FLAD1 gene and severe neuromuscolar disorders [4].

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Human apoptosis inducing factor: from its molecular mechanism to its functional and pathological significance

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Keywords: Apoptosis Inducing Factor, dimerization, NADH biding, partner interactions

The mitochondrial Apoptosis Inducing Factor (AIF) was firstly described as a caspase-independent death effector[1]. Upon an apoptotic stimulus, AIF is released from the mitochondrial intermembrane space, then translocating to the nucleus, where induces large-scale DNA fragmentation. Beyond its apoptotic function, AIF is a ubiquitous eukaryotic flavoprotein that is key for cell survival and for normal development. The NADH oxidoreductase activity of AIF was reported to be essential in the mitochondrial bioenergetics by indirect regulation of the biogenesis of major respiratory chain complexes[2]. In humans, mutations in AIF cause severe neurological disorders associated to OXPHOS failure and increase of its cytocidal potential[3,4]. Moreover, NADH binding modulates AIF monomer-dimer equilibrium in healthy mitochondria and could be determinant in its biological interactions with other molecules including a variety of protein partners[5]. Further characterization of the AIF molecular activity is key for a better understanding of the mechanism of the diseases in which it is involved and for exploring new therapeutic approaches. In this context, we are using a multidisciplinary methodology combining biochemical, biophysical and cellular techniques to understand the AIF physiological role in cellular death and life, as well as to identify compounds that might modulate these activities[5,6].

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Crystal structures and atomic model of NADPH oxidase

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Keywords: Membrane protein, Reactive Oxygen Species, Oxidative Stress, Redox Biology.

NADPH oxidases (NOXs) are the only enzymes exclusively dedicated to ROS generation. Dysregulation of these polytopic membrane proteins impacts the redox signaling cascades that control cell proliferation and death. We describe the atomic crystal structures of the catalytic FAD- and heme-binding domains forming the core subunit that is common to the seven members of the family. The domain structures were then docked *in silico* to provide a generic model of NOX family. A linear arrangement of cofactors (NADPH, FAD, and two membrane-imbedded heme moieties) injects electrons from the intracellular side across the membrane to a specific oxygen-binding cavity on the extra-cytoplasmic side. The overall spatial organization of critical interactions is revealed between the intracellular loops on the trans-membrane domain and the NADPH-oxidizing dehydrogenase domain. In particular, the C-terminus functions as a toggle switch, which affects access of the NADPH substrate to the enzyme. The essence of this mechanistic model is that the regulatory cues conformationally gate NADPH-binding, implicitly providing a handle for activating/deactivating the very first step in the redox chain. Such insight provides a framework to the discovery of much needed drugs that selectively target the distinct members of the NOX family and interfere with ROS signaling.

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The Biochemistry and Biophysics of D-arginine dehydrogenase from *Pseudomonas aeruginosa*

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Keywords: D-arginine dehydrogenase, amino acid oxidation, molecular dynamics, proton transfer.

D-Arginine dehydrogenase (*Pa*DADH, E.C. 1.4.99.6) from *Pseudomonas aeruginosa* is an FAD-containing enzyme that catalyzes the oxidation of D-arginine to 2-ketoarginine and ammonia[1-8]. *Pa*DADH is part of a two-enzyme system that catalyzes the racemization of D-arginine to L-arginine. The enzyme is a true dehydrogenase showing absolute lack of reactivity towards dioxygen. *Pa*DADH displays broad substrate specificity being able to oxidize all the standard D-amino acids except D-glutamate and D-aspartate. At pH 8.7, the second order rate constants k_{cat}/K_m span 7 orders of magnitude from 3.4 x 10^6 $M^{-1}s^{-1}$ with D-arginine to 0.75 $M^{-1}s^{-1}$ with D-threonine as a substrate. E87 in the active site of the enzyme dictates the specificity towards cationic substrates, as suggested by X-ray crystallography, mutagenesis, and pH profiles of the steady-state kinetic parameters of the wild-type and E87L variant enzymes. Mutagenesis studies have elucidated the role of the active site residues H48, Y53, and Y249, in either substrate binding or transition state stabilization. Removal of the hydroxyl group of Y249 has a minimal impact on the kinetic parameters of the enzyme, but results in the partial hydroxylation of the C6 position of the flavin with consequent inactivation of the modified enzyme. This underscores the importance of active site residues for the tight control of the flavin versatility beyond their typical roles in substrate binding and catalysis. A combination of crystallography, kinetic, computational, and mutagenesis data on S45 and A46 located in the FAD-binding domain portion of loop L1 demonstrated the importance of loop dynamics in substrate capture and catalysis. Solvent and substrate kinetic isotope effects with the slow analog D-leucine as a substrate for the wild-type enzyme established an asynchronous hydride transfer, which is triggered by the removal of a proton from the α-NH_3^+ of the amino acid substrate. The proton transfer is restricted with D-lysine as a substrate for the wild-type enzyme and D-arginine in select active site variants, i.e., S45A, A46G, H48F, and E87L, as suggested by the presence of a hollow in the log(k_{cat}) pH-profiles. *Pa*DADH is emerging as a paradigm for flavin-dependent enzymes that oxidize cationic D-amino acids and is an interesting complement to the well-characterized D-amino acid oxidase, which oxidizes small, hydrophobic substrates.

This work was supported in part by National Science Foundation grants CHE-1506518 (G.G.), MCB-1517617 (D.H.), and the Georgia Research Alliance. J.B. was supported by a Georgia State University Molecular Basis of Disease Fellowship; M.S. was supported by a U.S. Department of Education GAANN Fellowship and is a Georgia State University Bio-Bus Fellow.

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Activation of the class Ib ribonucleotide reductase by a flavin network in *Bacillus cereus*

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Keywords: enzyme activation, flavodoxin reductase, ribonucleotide reductase, structure.

Several enzyme systems are activated through flavodoxin / flavodoxin reductase pathways. For instance, in the class Ib ribonucleotide reductases (RNRs) the flavodoxin-like protein NrdI is essential in one of the redox networks that together activate the RNR enzyme system. RNRs reduce ribonucleotides to deoxyribonucleotides by employing radical chemistry, and NrdI activates the dimanganese center in the radical generating RNR β subunit NrdF. As seen by crystallography, the NrdI protein binds to NrdF, generating a gated channel between the FMN site in NrdI and the dimanganese center in NrdF, which allows for transfer of superoxide and activation of the RNR class Ib.[1]

It has been proposed that NrdI itself is recycled *in vivo* by an NrdI reductase, but no NrdI reductase has been identified. Flavodoxin-NADP+ oxidoreductases (FNRs) are probable reductants of NrdI. We identified three, thioredoxin-like flavodoxin reductases in the genome of *Bacillus cereus* and carried out structural and functional studies in order to characterise their ability to reduce NrdI. Binding studies showed that all three FNRs bind NrdI with similar affinities, however, steady-state kinetics revealed that one FNR reduces NrdI at a much higher rate than the other two FNRs.[2,3] Using this FNR as an NrdI reductase, we were also able to activate the NrdF under aerobic conditions, mimicking cellular conditions. Altogether, our observations suggest that this FNR might be the superior NrdI reductase *in vivo*, thereby completing the flavin activation network of class Ib RNR.[2] The activation and interaction between NrdI and FNR is likely linked to the observed rotation of the NADPH domains relative to the FAD domains in the *Bacillus cereus* FNR crystal structures.

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Kinetics and thermodynamics in ligands binding as FMN production determinants in bifunctional FAD synthetases

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Keywords: Riboflavin kinase, FAD synthetase, stopped-flow, ITC.

Prokaryotic bifunctional FAD synthetases (FADSs) exhibit ATP:riboflavin kinase (RFK) activity in its C-terminal module and FMN:ATP:adenylyltransferase (FMNAT) activity in the N-terminal one[1]. The RFK activity of the *Corynebacterium ammoniagenes* enzyme (*Ca*FADS) is highly regulated by RF substrate inhibition as well as by reaction products inhibition[2,3]. Contrary to *Ca*FADS, the homologous enzyme from the human pathogen *Streptococcus pneumoniae* (*Spn*FADS) does not show inhibition of its RFK activity and its FMNAT activity only works with the reduced FMN. Such dissimilarities suggest different mechanisms contributing to regulate the flavin and flavoproteome cellular homeostasis. Considering also the FADSs potential as exploitable targets in the design of selective inhibitors to treat infectious diseases, the comparative study of the RFK activity mechanisms in these two enzymes is a topic worthy of study.

Here we shed light on the regulation of the RFK activity of *Ca*FADS through the study of the role played by its substrates and products as inhibitors. In addition, the pre-steady-state kinetic and thermodynamic evaluation of binding of substrates and products to *Ca*FADS and to *Spn*FADS, collectively with the available structural information, allows depicting key differential aspects in the induced fit of substrates to reach the RFK catalytic complex. Our data reveal as the main cause for the inhibition in *Ca*FADS, the formation of all possible non-competent complexes with substrates and products, which compete, both kinetically and thermodynamically, with the catalytically competent one. Thus, a complex network of non-competent interactions provides with an intricate regulatory mechanism that allows the suitable production of flavins according to the *C. ammoniagenes* necessities. Such strategy agrees with selective inhibition of key enzymes being a common tool to regulate metabolic pathways. On the other hand, binding of the RFK reaction substrates to *Spn*FADS is by far the faster and more favorable process. Furthermore, in *Ca*FADS the Mg^{2+}-dependent-concerted allocation of substrates is required for catalysis, while a sequential binding order, also cation-dependent, is found in *Spn*FADS.

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Ultrafast kinetic studies reveal effects of electron transfer and charge recombination on the lifetime of flavin semiquinone

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Keywords: Transient Absorption Spectroscopy, Electron Bifurcation, Flavin, Electron Transfer.

Flavin-based electron transfer bifurcation is emerging as a fundamental and pervasive mechanism for conservation and deployment of electrochemical energy in enzymatic systems. It is believed that thermodynamic destabilization of a flavin semiquinone species is essential, so it is expected that this species should be short-lived. We now demonstrate that a short-lived anionic flavin semiquinone (ASQ) is not sufficient to infer bifurcating activity, although it may be necessary. To address this question, we have used transient absorption spectroscopy to compare the rates and mechanisms of decay of ASQ generated photochemically in bifurcating and non-bifurcating flavoproteins. We find that different mechanisms dominate in mediating decay of ASQ, producing lifetimes ranging over two orders of magnitude. Capacity for electron transfer among redox cofactors vs. charge recombination with nearby donors succeeds in explaining the range of ASQ lifetimes we observe. Our data support a model wherein efficient electron propagation can be an explanation for the short lifetime of the ASQ of bifurcating NADH-dependent ferredoxin-NADP+ oxidoreductase I, and a diagnostic ingredient of capacity for electron bifurcation.

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Flavins, flavoproteins and flavinologists

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An illustrated guide will be provided of the history of flavins, flavoproteins and those who studied them.

Poster Abstracts

(in alphabetical order)

Cryptochromes and perception of electromagnetic fields: role of the redox photocycle and potential signaling mechanisms

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Keywords: cryptochromes, photoreduction, Reactive Oxygen, magnetosensing

Cryptochromes are wide-spread evolutionarily conserved flavoprotein receptors with diverse sensory functions in organisms ranging from plants to humans. Cryptochromes are activated by a photocycle involving flavin photoreduction (1) and the biosynthesis of ROS (2,3). Though initially characterized as light sensors, cryptochromes have also been proposed as possible magnetosensors based on photochemical properties which are compatible with the Radical Pair Model of chemical magnetosensing (4). This suggestion is supported by increasing evidence of cryptochromes implicated in magnetosensing in multiple organisms.

In this presentation we show data using isolated proteins on the conserved photochemical characteristics of the cryptochrome photocycle that are compatible with signaling and radical pair formation. We then probe magnetic sensitivity *in vivo* under defined light and magnetic field conditions using the drosophila, plant, and migratory bird model systems to identify the most likely magnetosensitive step in the cryptochrome photocycle. Our results exclude the currently held hypothesis that magnetosensing by cryptochromes could occur in the course of light-induced forward electron transfer through the conserved Trp triad to the flavin radical, as proposed in numerous studies to date (5). Instead, magnetosensing occurs during the light-independent flavin reoxidation step, with some form of reactive oxygen species (ROS) as a likely intermediate.

Finally, we provide evidence for the first time that cryptochromes are involved in magnetic sensitivity to weak (low mT range) magnetic fields in mammalian and human cells, and that this cryptochrome magnetosensing mechanism also does not require light and involves the formation of ROS.

These results will be discussed with respect to the prevailing models of magnetoreception currently in the literature, as well as with respect to disturbing implications on the possible long-term effects of exposure to electromagnetic noise in the environment may have on public health.

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Structure, function and evolution of $F_{420}H_2$ utilizing flavin/deazaflavin oxidoreductases (FDORs) in Mycobacteria

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Keywords: deazaflavin(F_{420}), mycobacteria, oxidoreductases, biliverdin reductase

Mycobacteria produce a rare deazaflavin cofactor F_{420} found in Actinobacteria and Archaea, which is important for persistence during oxidative stress, including in pathogenic species like *Mycobacterium tuberculosis*. However, the mechanisms of F_{420} utilization in mycobacteria remain largely unknown. We have characterized the largest mycobacterial F_{420} utilizing protein family called the flavin/deazaflavin dependent oxidoreductases (FDORs), to identify their functional roles in aiding mycobacterial infection and persistence. This family also consists of proteins with cofactor specificities for FMN, FAD and heme. By solving five novel X-ray crystal structures of FDORs and comparing them with previously available structures, we showed that FDORs have relatively conserved cofactor binding regions and extensively modified substrate binding pockets for variable functions.

Their cofactor preference, sequence similarity and existing characterizations allowed classification into functional groups, including the $F_{420}H_2$-dependent quinone reductases that also activate 4-nitroimidazole pro-drugs approved for combating multi-drug resistant *M. tuberculosis*. Novel FAD binding proteins that could be involved in triggering mycobacterial dormancy were also discovered, and *in silico* substrate docking was used to identify $F_{420}H_2$-dependent fatty acid saturases and $F_{420}H_2$-dependent biliverdin reductases (F-BVRs). Characterization of the F-BVR Rv2074 from *M. tuberculosis* showed its ability to reduce biliverdin-IXα to bilirubin-IXα, which is a potent antioxidant that could aid *M. tuberculosis* survive oxidative stress encountered inside macrophages during infection. This $F_{420}H_2$-dependent biliverdin reduction by Rv2074 is mechanistically similar to the NAD(P)H-dependent reactions in the mammalian biliverdin reductases as inferred using the structure of the Rv2074:F_{420} complex with biliverdin modelled into the active site.

Lastly, we showed that F_{420} production is more widespread in the bacterial community than previously thought, where Proteobacteria and Chloroflexi also produce F_{420} and encode FDORs for its utilization. Overall, this body of work characterized novel flavin/deazaflavin proteins, aiding to uncover the role of the rare deazaflavin cofactor F_{420} in bacterial metabolism. 2

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Structural bases of the altered functional properties of a pathological variant of the apoptosis inducing factor (AIF)

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Keywords: neurodegeneration, mitochondria, charge-transfer complex, ligand binding.

The apoptosis inducing factor (AIF) is a mitochondrial NAD-dependent flavoprotein, which, in addition to its long-known function in apoptosis, plays an essential role in respiratory chain biogenesis and maintenance, possibly *via* its interaction with CHCHD4/MIA40.1 Twenty human allelic variants of AIF are currently known as the cause of different neurodegenerative diseases. A strong correlation has been observed between the loss of ability of the pathological AIF forms to stabilize the charge-transfer (CT) complex between FADH- and NAD+ and the severity of the resulting illnesses, pointing to a pivotal role played by its redox activity and the related monomer-dimer transition of AIF in its physiological function.2 Recently, we demonstrated that the G307E replacement in murine AIF (equivalent to the pathogenic G308E in the human protein) dramatically decreases the rate of CT complex formation by hampering AIF interaction with NAD+/H.3

Here we report the crystal structures of murine AIF-G307E in both its oxidized and CT complex states. From their comparison with the corresponding structures of wild type AIF, we ascertained how the increased steric hindrance of the replacing side chain leads to changes in the network of protein-ligand contacts. In particular, the G307E mutation causes the disruption of the interactions that, in the wild-type protein, Glu335 and Lys341 establish with the adenosine ribose and the pyrophosphate of NAD+, respectively. This in turn results in a 2 Å shift and reorientation of the adenylate moiety of bound NAD+, which appears to be the main cause of the observed decrease in the rate of CT complex formation. Moreover, we found that the altered balance between the binding of the adenine/nicotinamide moieties of the coenzyme determines a large drop in AIF-G307E ability to discriminate between NADH and NADPH.

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Formation and decay of the flavin 4a-hydroperoxide of substrate-free *p*-hydroxybenzoate hydroxylase

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Keywords: *p*-hydroxybenzoate hydroxylase, flavin 4a-hydroperoxide, pulse radiolysis

Flavoprotein monooxygenases form flavin C(4a)-hydroperoxides (P-*Fl*-OOH) during enzyme turnover. Hydroperoxides are also inferred in the activity of flavoprotein oxidases, but not observed in enzyme reactions due to rapid decay yielding oxidized flavoprotein and H_2O_2. The pulse radiolysis technique (~60 Gy in 2 μs) combined with time-resolved spectrophotometry has been instrumental in characterizing the spectral features and lifetime of the radiolytically generated *Fl*-OOH of glucose oxidase in the absence of substrate[1]. Radical reactions in N_2O/O_2-saturated aqueous solutions containing formate ions produce quantified yields of the one-electron reductant, $CO_2^{\bullet-}$, which rapidly reduce (≤ 10 μs) both the protein and O_2. The neutral flavosemiquinone thus formed (P-*Fl*H•, ~2 μM) undergoes a pseudo 1st-order reaction (1) with the large excess concentration of superoxide (~35 μM) to form P-*Fl*-OOH, followed by its decay, (2).

$$O_2^{\bullet-} + \text{P-}Fl\text{H}\bullet + H^+ \rightleftharpoons \text{P-}Fl\text{-OOH} \qquad K = k(1)/k(-1) \qquad (1)$$

$$\text{P-}Fl\text{-OOH} \rightarrow \text{P-}Fl + H_2O_2 \qquad (2)$$

Using the above procedure we have previously produced the flavin hydroperoxide of the monooxygenase, *p*-hydroxybenzoate hydroxylase (PHBH). While the spectral features of the PHBH hydroperoxide are similar to those of free *Fl*-OOH and substrate-free glucose oxidase[1], the rate constant of $O_2^{\bullet-}$ with P-*Fl*H• of PHBH, $k(1) = 4 \times 10^7$ M^{-1} s^{-1}, is considerably smaller than with glucose oxidase of 8.5×10^8 M^{-1} s^{-1}. Furthermore, the PHBH *Fl*-OOH kinetically stabilized by the protein to a greater degree than seen with glucose oxidase, decaying with a rate constant of $k(2) = 64 \pm 9$ s^{-1} *c.f.* 340 ± 20 s^{-1}. Varying the concentration of superoxide with the radiation dose and constructing kinetic plots, both protein *Fl*-OOH species are found to form equilibria with superoxide, where $k(-1)$ for glucose oxidase is 5800 ± 1200 s^{-1} and $k(-1)$ for PHBH is 440 ± 20 s^{-1}. The greater stability of P-*Fl*-OOH in the monooxygenase compared to the oxidase may well reflect the differing reactivities of the proteins towards substrates.

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Quinones and nitroaromatic compounds as subversive substrates for *S. aureus* flavohemoglobin

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Keywords: Flavohemoglobins, *Staphylococcus aureus*, quinones, nitroaromatic compounds

In microorganisms, flavohemoglobins (FHbs) containing FAD and heme (Fe^{3+}, metHb) convert $NO^{\cdot}$ into nitrate at the expense of NADH and O_2. FHbs contribute to bacterial resistance to nitrosative stress. Because FHbs are not present in mammals, the inhibition of FHbs or a subversion of their functions may be an efficient approach to decrease pathogen virulence. Inhibitors of FHb such as econazole, ketoconazole, and miconazole were shown to bind as the sixth ligand to the heme iron[1] and their binding modulates the FHb-catalyzed formation of anion superoxide[2]. In this work, we have studied the reactivity of FHbs with quinones and nitroaromatic compounds. Our data show that *S. aureus* FHb rapidly reduces these compounds by a mixed single- and two-electron pathway. The reactivity of nitroaromatics increased upon an increase in their single-electron reduction potential (E^1_7), whereas the reactivity of quinones poorly did with strong preference for the 2-OH-1,4-naphthoquinone structure. The reaction followed 'ping-pong' mechanism. In general, the maximal reaction rates were lower than the maximal presteady-state rate of FAD reduction and/or oxyhemoglobin ($HbFe^{2+}O_2$) formation at the expense of NADH (~130 s^{-1}, pH 7.0, 25 ^{o}C), indicating that the enzyme turnover is limited by the oxidative half-reaction. The enzyme turnover studies showed that quinones preferentially accept electrons from reduced FAD, and not from $HbFe^{2+}O_2$. These results suggest that quinones and nitroaromatics act as 'subversive substrates' for FHb, and may enhance the cytotoxicity of $NO^{\cdot}$ by formation of superoxide and diverting the electron flux coming from reduced FAD. Because quinone reduction was activated by the inhibitors of FHb, econazole, ketoconazole, and miconazole, their combined use may represent a novel chemotherapeutical approach.

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Bacteria are constantly exposed to a diverse range of oxidative stresses caused by a range of reactive species in the form of antibiotics, their natural biome as well as the interplay between their hosts. Many organisms have a distinct way in which they are able to counteract these oxidative stresses. In the case of bacteria, redox homeostasis and thiol-disulphide reactions represent a diverse network of interesting reactions that are necessary in their maintenance, regulation and survival.

Unlike most other organisms, the multi-drug resistant pathogen *Staphylococcus aureus* (MRSA) does not use glutathione (GSH) as low molecular weight thiol as part of its mechanisms to resist oxidative stress and maintain redox homeostasis. Instead, it has high levels of the thiol-containing cofactor coenzyme A (CoA) and a concomitant CoA disulfide reductase flavoenzyme that can recycle this cofactor, although the relevance of this system in oxidative stress resistance has not been demonstrated as yet. However, this has been done for bacillithiol (BSH) a thiol metabolite uniquely found in certain Gram-positive bacteria. Yet, it remains unclear how BSH is recycled as no BSH disulfide reductase has been characterized.

Previous studies have identified a putative pyridine nucleotide disulfide oxidoreductase (PNDOR) enzyme as being >80-fold upregulated in *S. aureus* upon phagocytosis by neutrophils, which use oxidative stress challenge as part of their killing mechanisms. This raised the question whether this flavoprotein, which shows homology to mercuric ion reductases, fulfils another role other than its putative assignment. Here we report the characterization of the PNDOR MerA in *S. aureus* and the possible role of the enzyme in oxidative stress resistance in this organism.

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Enzymology of H_2S oxidation in nanodiscs

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Keywords: sulfide, nanodiscs, charge-transfer, CoQ.

The mitochondrial sulfide oxidation pathway is critical for regulating levels of hydrogen sulfide (H_2S), which plays critical signaling roles in mammals leading to cardioprotection, neuroprotection, and anti-inflammatory responses in the gastrointestinal system. The first and committed step of this pathway is catalyzed by sulfide-quinone oxidoreductase (SQR), a membrane-bound, flavin-dependent disulfide reductase. SQR converts H_2S to an enzyme-bound cysteine persulfide and subsequently transfers the sulfane sulfur to a small molecule acceptor (Fig 1). Concomitant with the latter step, the cysteine disulfide reforms in the active site while the electrons from sulfide oxidation are relayed to the FAD cofactor, to coenzyme Q_{10}, and thereon to complex III in the electron transport chain, thus coupling sulfide oxidation to mitochondrial energy metabolism. While the pivotal role of SQR in H_2S clearance makes it an attractive therapeutic target in diseases attributed to dysregulated H_2S levels, key features of the SQR reaction mechanism are unclear and studies have been hindered by the limited solubility of this membrane-bound enzyme. In the present study, we have elucidated the enzymology of human SQR in a membrane environment by incorporating it into nanodiscs. Kinetic analyses of the nanodisc-bound SQR reveal enhanced activity with several small-molecule acceptors compared to the detergent-solubilized form of the enzyme, while stopped-flow studies further elucidated the pre-steady state catalytic mechanism and the kinetics of FAD-mediated quinone reduction. This study provides the kinetic framework for assessing the SQR reaction in the context of the mitochondrial sulfide oxidation pathway.

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Structural studies of human monoamine oxidase B in complex with potent chromone-based inhibitors

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Keywords: monoamine oxidase, inhibitor, Parkinson's disease, drug design.

Monoamine oxidases A and B (MAOA and MAOB) are flavoenzymes bound to the outer-mitochondrial membrane which catalyze the oxidative deamination of arylalkylamine neurotransmitters such as serotonin, adrenaline and dopamine[1]. They represent established neurological drug targets and very recently safinamide, a reversible potent MAOB-specific inhibitor, was approved as a new anti-Parkinson treatment[2]. A new role of MAOs outside the central nervous system is emerging which highlights these enzymes as drug targets, more specifically in relation to the molecular mechanisms underlying cardiac ageing[3] and to prostate cancer progression[4]. Among the many MAO inhibitors, limited data are available on the chromone-based compounds. Recently a series of analogs of this class of compounds was developed which potently inactivate MAO B with K_i values in sub-nanomolar range [5]. Structural studies on human MAO B in complex with these inhibitors will be presented which reveal the exact mechanism of inhibition and unravel active site conformational changes specifically observed with this class of ligands.

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Insights from variation of substrates and protein platforms in nitroreductase

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Keywords: nitroreductase, flavoenzyme, enzyme-aided semisynthesis, Hammett analysis.

Nitroreductases (NRs) found in all three kingdoms of life employ a flavin to mediate 2-electron redox catalysis. The NRs of bacteria transform a variety of nitrated aromatics and play critical roles in therapeutic prodrug activation as well as deleterious activation of xenobiotics. To inform deployment of this versatile reactivity to reduce nitroaromatics to the corresponding amines we have compared two contrasting NRs with respect to substrates spanning a range of reduction potentials. Substrates with favourable enthalpies for hydride acquisition are 100 x more rapidly reduced by both the *Mycobacterium smegmatis* (*Ms*) and *Salmonella typhimurium* (*St*) NRs. However, even the substrates with the most electron withdrawing substituents did not produce amines from simple substrates. Nor did the enzyme with a published record of amine production support more rapid nitro-reduction. Only two substrates yielded amine product and the two enzymes displayed similar k_{cat}/K_M values and trends vs. pH. However *St*NR's k_{cat} was 20 times higher than *Ms*NR's and displayed pH dependencies not evident for *Ms*NR. Crystal structures show that the substrate binding sites of *St*NR and *Ms*NR are constrained by distinct active site 'lid' subdomains inserted into different locations of the NR core sequence, and analysis of representative NR structures revealed that NRs related to *E. coli*'s NsfA share a third distinct active site lid while a fourth group of NRs lacks a lid. These differences may explain the distinct substrate specificities of NRs.

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Genomic integration of *luxF* into the *Vibrio harveyi lux* operon

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Keywords: bacterial bioluminescence, luciferase, myrFMN, *luxF* knock-in

Bioluminescence is the enzymatic production of light by living organisms. In bioluminescent bacteria the heterodimeric enzyme luciferase catalyzes the monooxygenation of long-chain aliphatic aldehydes to the corresponding acids utilizing FMN as redox cofactor. The free energy released during this oxidation causes an excited state FMN-4a-hydroxide, which serves as the light emitting luciferin.[1] All proteins involved in bacterial bioluminescence are encoded by the *lux* operon - *luxCDABEG*. The *luxA* and *luxB* genes encode for the α and β subunits of luciferase; *luxC*, *luxD*, and *luxE* are part of a fatty acid reductase complex and *luxG* encodes for a flavin reductase. Many strains in *Photobacteria* carry the additional gene *luxF*. LuxF is a homodimeric protein, which can bind the unusual flavin derivative 6-(3'-(*R*)-myristyl)-FMN (myrFMN) but its distinct role is still unresolved.[2]

It was postulated that myrFMN is a potential inhibitor of the luciferase. Due to a higher affinity of myrFMN to LuxF than to luciferase, we hypothesized that LuxF is scavenging myrFMN to prevent inhibition.[2] Replication of the bioluminescent reaction *in vitro* proved that myrFMN is produced by the luciferase itself. *In vivo* analysis of different bioluminescent bacteria (*luxF*$^+$ and *luxF*$^-$) revealed that myrFMN formation is independent of *luxF* occurrence but there seems to be a positive correlation between light intensity and myrFMN formation.[3]

This finding suggests that *luxF* influences the light production in bacterial bioluminescence. Thus we expect to shed light on the role of *luxF* by genomically engineering a *luxF*$^-$ strain of *Vibrio harveyi*. Therefore, we constructed an integration vector harboring the *luxF* gene of *Photobacterium mandapamensis* that will enable us to insert the *luxF* gene into the genome of *Vibrio harveyi*. Subsequently, wild-type *Vibrio harveyi* (i.e. *luxF*$^-$) will be compared to the *luxF* knock-in (*luxF*$^+$) strain to further investigate the role of *luxF* in bacterial bioluminescence.

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Mass spectrometric analysis of 4-hydroxy-2-nonenal adduction to electron transfer flavoprotein

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Keywords: electron transfer flavoprotein, 4-hydroxy-2-nonenal, mass spectrometry, reactive oxygen species

The formation of protein adducts by lipid peroxidation by-products of oxidative stress changes the function and conformation of the protein. In this study, the adduction of electron transfer flavoprotein (ETF) by the reactive aldehyde 4-hydroxy-2-nonenal (4HNE) is investigated. In the mitochondrial matrix, ETF is a soluble heterodimeric protein that accepts electrons from a number of acyl-CoA dehydrogenases and donates them to membrane bound electron transfer flavoprotein-ubiquinone oxidoreductase (ETF:QO). Previous studies have shown that 4HNE reduces the activity of ETF with evidence suggesting that 4HNE crosslinks the two ETF subunits.[1] These crosslinks could reduce the flexibility of the ETF subunits and affect electron transfer. To ascertain the molecular nature of the effects of 4HNE on ETF, tryptic digests of ETF were incubated with 4HNE and evaluated using high resolution liquid chromatography-mass spectrometry (LC-MS). Custom synthetic polypeptides containing ETF sequences were also incubated with 4HNE to test reactivity and optimize the LC-MS analyses. Mass spectral data in combination with database searches indicate 27 possible 4HNE adduction sites on ETF of which 13 are located near the exterior. Amino acids of particular interest are His-88 and His-286 which show strong affinity for 4HNE and are located near the FAD cofactor. Five possible sites of ETF cross-linking are also indicated by the mass spectral data. Additionally, results of preliminary computational studies of 4HNE reactions with cysteine, histidine, and lysine in the context of ETF peptide sequences will be reported.

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Inhibition studies of the dihydroorotate dehydrogenase from *S. mansoni* using antimalarial drugs repositioning

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Keywords: dihydroorotate dehydrogenase, flavoenzymes, drug repositioning, inhibition studies.

Drug repositioning consists in evaluating or using existing drugs for the treatment of diseases other than those for which they were originally developed.[1] Schistosomiasis, also known as snail fever, is a parasitic disease caused by blood flukes of the genus Schistosoma. In terms of impact, this disease is second only to malaria as the most devastating parasitic disease and up to this moment, only one medication is known and applied: Praziquantel. Although this drug has been widely and effectively used for many years, some studies have already shown resistant organisms towards it.[2,3] That is the reason we chose this disease as focus of study to repurpose drugs. One of the approaches to the development of new drugs is through the inhibition of enzymes present in important biochemical pathways. *S. mansoni* possesses all six enzymes in the *de novo* biosynthesis of pyrimidine, including the enzyme dihydroorotate dehydrogenase, DHODH, a flavoenzyme that contains FMN as a cofactor. The reaction catalyzed by DHODHs is the fourth and the only redox reaction in this biochemical pathway. Due to the importance of pyrimidine biosynthesis pathway, the fact that the DHODHs from *S. mansoni* and *Plasmodium falciparum* share high similarities and the latter already has known inhibitors which are already in clinical studies and/or in the market, we decided to evaluate the repurposing of malarial DHODH inhibitors against *Sm*DHODH. In our studies inhibitors were identified with great inhibitory potential (low micro molar scale). A number of derivatives from one in-market antimalarial drug were synthetized. Those derivatives with better potential (low nanomolar scale) had their inhibition mechanisms performed, in which, competitive, non-competitive and mixed inhibition mechanisms were found. Pharmacophore model was also performed, giving important insights on how to improve such molecules for obtaining lead compounds.

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D-amino acid oxidase mutations related to human diseases

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Keywords: D-amino acid oxidase, D-serine metabolism, NMDAR mediated neurotransmission, pathological dysfunction.

In human brain, D-amino acid oxidase (DAAO, EC 1.4.3.3) catalyzes the degradation of D-serine (D-Ser), the main co-agonist for the activation of NMDA receptors (NMDAR). A relationship between D-serine signalling deregulation, NMDAR dysfunction and diseases of central nervous system is widely assumed. In particular, a decreased concentration of D-Ser is associated with psychiatric diseases such as schizophrenia while increased levels are related to neurodegenerative disorders such as amyotrophic lateral sclerosis (ALS).

Various SNPs of the human *DAAO* gene resulting in non synonymous substitutions have been reported. We expressed in *E. coli* and characterized several DAAO variants. In order to clarify the role of human DAAO on D-Ser cellular concentration, we propose a functional classification of known substitutions based on biochemical properties of enzyme variants related to SNPs.

The D31H, W209R and R279A DAAO variants possess an increased kinetic efficiency and FAD affinity and result into a decreased D-Ser level when overexpressed in human glioblastoma U87 cells [1, 2], thus suggesting an involvement in schizophrenia susceptibility (i.e. hypoactivation of NMDAR-mediated neurotransmission).

The R199W (associated with a familial ALS) and R199Q DAAOs possess a significant decrease of the enzymatic activity and a lower affinity for FAD, and the G183R variant is fully inactive: these variants produce an increase of D-Ser concentration in U87 cells [2]. Moreover, the expression of G183R or G331V DAAO variants in U87 cells induces formation of protein aggregates that colocalize with ubiquitin [1]. According to the lower kinetic efficiency and the protein instability, these latter DAAO variants seem to be involved in pathologies related to D-serine induced cytotoxicity such as in neurodegenerative pathologic processes.

In conclusion, our studies are aimed to clarify the role of human DAAO on D-Ser cellular concentration and NMDAR-dependent neurotransmission under both physiological and pathological conditions.

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ITC as a valuable method to study reaction kinetics and inhibition of human flavin-containing monooxygenase 3

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Keywords: ITC, KINETICS, FMO, TMAO

Human flavin-containing monooxygenase 3 (hFMO3) is a phase I drug metabolizing enzyme that is also responsible for the physiological transformation of trimethylamine to trimethylamine N-oxide (TMAO). Recent reports highlighted a clear connection between TMAO, atherosclerosis and cardiovascular disease placing hFMO3 in the spotlight for the identification of suitable molecules that can inhibit its function. hFMO3 was studied using Isothermal Titration Calorimetry (ITC) to characterize the reaction kinetics for NADPH, trimethylamine and two other well known substrates benzydamine and ethionamide. Both single-injection and multiple-injection ITC modes were employed to determine the kinetic parameters k_m and k_{cat} for the selected substrates. Temperature and pH dependence of for S- and N- oxidation reactions were also studied together with the intrinsic enthalpy of the reaction (ΔH_{int}). Finally, a well known inhibitor of hFMO3, methimazole, was tested for its ability to compete for the oxidation of TMA. The results were validated by comparing the kinetic parameters to the previously published data on hFMO3. ITC proved to be a valuable method to study hFMO3 activity and to test inhibitors of the monooxygenation reaction that leads to the formation of the TMAO metabolite.

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Role of the flavin assembly factor SdhE in formation of the covalent flavin linkage in complex II

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Keywords: Succinate dehydrogenase, fumarate reductase, complex II, covalent FAD

Complex II (succinate-ubiquinone oxidoreductase, SQR or SDH) is a membrane-bound heterotetramer found in the mitochondrial or bacterial inner membrane. More than fifty years ago the flavoprotein subunit (SdhA) was the first example of a protein found to contain a covalently bound flavin1. The formation of the covalent flavin bond was thought to be by an autocatalytic process2, however, in the past decade this was questioned when an additional protein (SdhAF2/SdhE) was shown to be necessary for covalent flavinylation in complex II3,4. Using a combination of mutagenesis, *in vivo* and *in* vitro photoaffinity cross-linking, mass spectrometry, x-ray crystallography and small angle x-ray scattering (SAXS) we investigated the role of the assembly factor in covalent flavinylation. Using *Escherichia* coli complex II and its related homolog fumarate reductase (FRD) we found a specific domain of the SdhA/FrdA flavoproteins that bound the SdhE assembly factor. The region of SdhA/FrdA bound to the assembly factor is near where the iron-sulfur protein (SdhB/FrdB) binds to the flavoprotein in the membrane-bound heterotetramer. Additional findings show that the redox state of the organism was also a factor into whether the assembly factor was needed for formation of the covalent flavin. SdhE appears to be needed to clamp the capping domain of the flavoprotein into a closed conformation to align specific amino acid residues near the catalytic site and isoalloxazine ring. This specific conformation allows the proposed quinone-methide self-catalytic reaction to proceed inducing formation of the covalent flavin bond. Additionally, interaction of the assembly factor with the flavoprotein stabilizes both proteins from proteolysis prior to assembly into the membrane-bound complex. Our data support the original quinone-methide self-catalytic mechanism for covalent flavin formation. The data also show that the assembly factor acts as a covalent flavin enhancing but non-essential chaperone.

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Kinetic properties of NfsA from *E. coli* l

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Keywords: nitroreductase, quinones, nitroaromatic compounds, kinetics

NfsA, a major FMN-associated nitroreductase of *E. coli*, reduces nitroaromatic compounds via consecutive two-electron transfers. NfsA has potential applications in the biodegradation of nitroaromatic environment pollutants, *e.g*. explosives, and is also of interest for the anticancer strategy gene-directed enzyme prodrug therapy. However, the catalytic mechanism of NfsA is poorly characterized. Here we examined the NADPH-dependent reduction of quinones (n = 16) and nitroaromatic compounds (n = 12) by NfsA. We confirmed a general "ping-pong" reaction scheme, and preliminary rapid reaction studies of the enzyme reduction by NADPH showed that this step is much faster than the steady-state turnover number*, i.e.,* the enzyme turnover is limited by the oxidative half-reaction. The reactivity of nitroaromatic compounds (log k_{cat}/K_m) followed a linear dependence on their single-electron reduction potential ($E1_7$), indicating a limited role for compound structure or active site flexibility in their reactivity. The reactivity of quinones was lower than that of nitroaromatics having similar $E1_7$ values, except for the significantly enhanced reactivity of 2-OH-1,4-naphthoquinones, consistent with observations previously made for the group B nitroreductase of *Enterobacter cloacae*1. We present evidence that the reduction of quinones by NfsA is most consistent with a single-step (H-) hydride transfer mechanism. NfsA catalyzes stoichiometric oxidation of NADPH by high-potential quinones. However, the enzymatic oxidation of NADPH by TNT (2,4,6-trinitrotoluene) and tetryl (2,4,6-trinitrophenyl-*N*-methylnitramine) was characterized by 2:1 and 4:1 stoichiometry, respectively, which was changed into 1:1 and 2:1 in the presence of ascorbate. It shows that TNT and tetryl are reduced into mono- and dihydroxylamine products, respectively. The inhibition of reaction by ascorbate is caused by its reduction of nitroso intermediate(s) of reaction, which oxidize NADPH directly.

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Engineering of pyranose 2-oxidase for expanding substrate utilization

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Keywords: pyranose 2-oxidase, enzyme engineering, sugar oxidation

Pyranose 2-oxidase (P2O) is an enzyme originally found in wood-decomposing fungi and belongs to the Glucose-Methanol-Choline (GMC) oxidoreductase family (1). P2O contains a covalently-linked flavin adenine nucleotide (FAD) as a cofactor and catalyzes oxidation of several aldopyranoses by molecular oxygen at the C2 position to yield the 2-keto-aldoses and hydrogen peroxide (2). Because P2O shows high specificity toward aldopyranoses, this property makes the enzyme attractive as a biocatalyst. Therefore, in this work, we explored catalytic power of P2O in sugar oxidation and also expanded its biocatalytic utilization through enzyme engineering. Pre-steady state kinetics of V546C with glucose and galactose, V546P with glucose and galactose, Y456W with glucose and mannose, T169G with galactose and arabinose, and C170A with all monosaccharides indicate that rate constants of the flavin reduction in these reactions are greater than those of wild-type enzyme reactions with the same substrates. Thermal stability investigation using the thermofluor method found that P2O(WT) and variants showed similar thermal stability with their melting points (T_m) ~52-54 °C. Therefore, V546C, V546P, Y456W, T169G, C170A are good variants that have broader substrate utilization than the wild-type enzyme and may potentially be used as biocatalysts for keto-sugar synthesis. Based on substrate and product analysis using GC/MS and GC/FID, multiple turnover reactions of wild–type P2O can be applied to completely convert 20 mM glucose, galactose and xylose to keto-sugars. Activity assays showed that the wild-type P2O was very stable and remained active in multiple turnover reactions at 25°C for 24 h.

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Rational design of flavins for organic photoredox catalysis: from photolyase models to effective tool in organic synthesis

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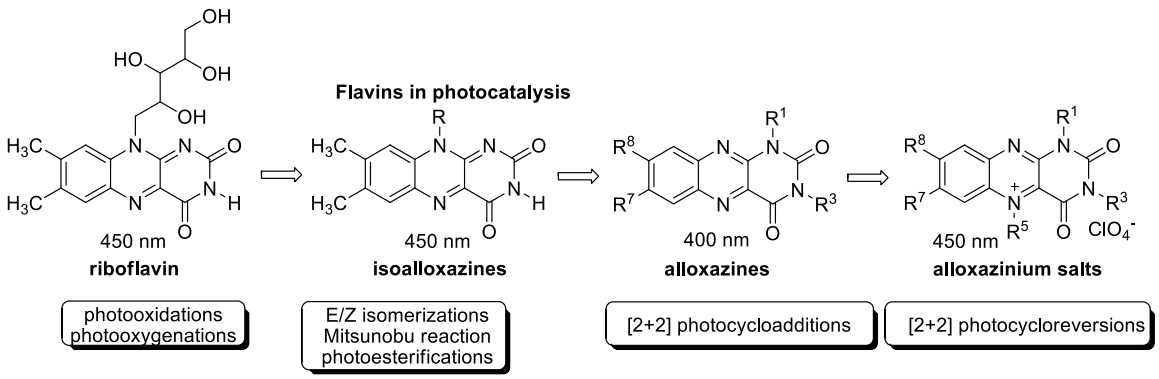
Keywords: Photocatalysis, Mitsunobu esterification, Photooxidation, Cycloreversion.

Photoexcitation allows chemical transformations that are not accessible by conventional methods. Use of visible light combined with a photocatalyst even improves classical photochemical methodologies avoiding side reactions of functionalities sensitive to UV light and making photochemistry available for most laboratories. Nowadays, photo-redox catalysis with organic photocatalysts[1] (organic photo-redox catalysis, photoorganocatalysis) is of a growing interest because of low costs of organic dyes and different reactions that they are able to mediate thus expanding the boundaries of organic synthesis.

One of the most prominent natural chromophores is represented by flavin cofactors which are involved in several light-dependent processes, for example, in light generation by bacterial luciferase, in plant phototropism and in photolyases in the cleavage of cyclobutane-pyrimidine dimers to repair DNA damage. Due to interesting photochemical properties, flavins have been tested in photocatalysis; nevertheless, their applications, except of photolyase models,[2] have been for a long time limited to photooxidations of benzylic derivatives.[3] In the presentation, recent flavin-based photocatalytic procedures will be discussed, especially visible light [2+2] photocycloaddition[4] and photocycloreversion,[5] photooxygenation,[6] photocatalytic Mitsunobu reaction,[7] esterification and Apple reaction. Special attention will be paid to rational design of flavin photocatalysts (see Scheme for illustration).



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Light and redox sensing by flavoproteins

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Keywords: cryptochromes, LOV-domain proteins, transmembrane signaling, photoreduction

Blue-light sensing proteins coordinate many biological processes that include phototropism, photomorphism, stress responses, virulence and the entrainment of circadian clocks. Three major types of blue-light sensors all bind flavin nucleotides as chromophores, but the photochemistry employed and conformational responses invoked differ considerably among the classes. Nevertheless, photoinduced electron transfer reactions play a key role in several mechanisms. How such reactivity leads to conformational signaling will be discussed for both cryptochromes (CRYs) and light- oxygen- voltage (LOV) domains. In CRYs, blue-light mediated flavin reduction promotes proton transfer within the active center that then leads to displacement of a key signaling element. For LOV proteins, blue light causes formation of a covalent cysteinyl-flavin adduct, which rearranges hydrogen bonding and restructures the N-terminal region of the protein. Interestingly, a new class of LOV-like sensor does not undergo adduct formation and instead can operate by flavin photoreduction, like CRY. In yet another related system, flavin reduction of a LOV-like domain serves to regulate transmembrane signaling. Conserved aspects of reactivity in these proteins provide lessons for the design of new photosensors, which may find use as tools in optogenetics.

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Novel flavoproteins of medical and ecological relevance

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Keywords: Flavoprotein, L-amino acid oxidase, Fumarate reductase, *Wolinella succinogenes*.

The organic cofactor flavin adenine dinucleotide (FAD), usually engaged in redox reactions, is the common feature connecting two novel enzymes, the amino acid oxidase APIT (*Aplysia punctata* ink toxin) with antitumor and bactericidal properties and the methacrylate reductase FccA from *Wolinella succinogenes* that is of high biotechnological interest for degrading methacrylate, a by-product of industrial plastic production.

APIT, isolated directly from the ink expelled by the marine sea hare *A. punctata,* is a 60 kDa FAD-containing L-amino acid oxidase with high substrate specificity. It catalyzes the oxidative deamination of L-lysine and L-arginine (that are frequently enriched in tumor cells) with concomitant production of cytotoxic hydrogen peroxide (H_2O_2), ammonium (NH_4^+) and the corresponding α-keto acid.

The flavoprotein FccA from *W. succinogenes* displays amino acid sequence homologies to flavocytochrome *c* fumarate reductases (FR) and to the catalytic subunit of the membrane-bound succinate:quinone oxidoreductase (respiratory complex II). This 56 kDa periplasmic flavoprotein is co-transcribed with FccB (a 16 kDa protein with homologies to the tetraheme domain of FR) and FccC (a membrane anchored tetraheme *c*-type cytochrome with homologies to the NirT/NapC family). Therefore, it is likely that FccA, FccB and FccC function as a membrane-associated electron transport chain, resulting in a periplasmic oxidoreductase complex. Interestingly, despite homologies to fumarate reductases, FccA is not capable to reduce fumarate, but instead the related methacrylate, a significant contaminant in industrial waste waters.

We have cloned FccA, FccABC and APIT, from codon optimized synthetic genes and heterologously expressed the proteins in *E.coli*. Purification of the proteins for X-ray structural evaluation is currently being pursued.

Berberine bridge enzyme-like proteins: from characterization to application

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Keywords: Berberine bridge enzyme-like proteins, monolignol oxidoreductase, vanillyl-alcohol oxidase flavoprotein family, lignin

Flavoproteins are a diverse protein class employing an isoalloxazine ring for catalysis in form of the flavin mononucleotide (FMN) or the flavin adenine dinucleotide (FAD). Among them, is the berberine bridge enzyme-like (BBE-like) protein family (pfam 08031) that was named after the berberine bridge enzyme (*Ec*BBE) from California poppy (*Eschscholzia californica*). BBE-like proteins form a multigene family in plants and the number of members varies from one in the moss *Physcomitrella patens* to 28 in Arabidopsis thaliana and 57 in the Western Balsam Poplar (*Populus trichocarpa*). Despite of the frequent occurrence of these proteins their function is largely unknown. Therefore, we chose to investigate the BBE-like proteins occurring in *A. thaliana* to broaden our understanding of this protein family. Presented will be the structural and biochemical characterization of *At*BBE-like protein 15 and 28, the application of an engineered *At*BBE-like 15 variant as biocatalyst, the role of *At*BBE-like 15 in plant physiology and the development of the BBE-like protein family in the plant kingdom.

Our analysis of the *At*BBE-like protein family reveals that there are four frequently occurring active site types in plants1,2. *At*BBE-like protein 15 is a representative of the most abundant type, therefore it can serve as a paradigm for the majority of these proteins. The enzyme was identified as monolignol dehydrogenase, an activity that was not recognized for this protein family before. Additionally, the enzyme was rationally engineered towards higher oxygen reactivity and the potential of the enzyme as biocatalyst for oxidative reactions was tested3. Experiments with *At*BBE-like 15 knock out plants revealed a perturbation of the content and the total amount of lignin in these plants. This indicates that monolignol dehydrogenases are responsible for the manipulation of the monolignol pool and represent an electron delivery system to fuel the lignification that was previously not recognized.

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The solid-state photo-CIDNP effect in aureochrome LOV-C287S studied by ^{13}C MAS NMR

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Keywords: Solid-state Photo-CIDNP, Aureochrome LOV C287S, FMN

Flavins are widely used dyes in liquid-state NMR to generate non-equilibrium nuclear spin polarization, detected as significantly enhanced absorptive or emissive signals upon illumination. This effect, known as photo-chemically induced dynamic nuclear polarization (photo-CIDNP), overcomes the intrinsically low sensitivity in magnetic resonance methods and find wide applications in the study of protein structures. The solid-state equivalent, the solid-state photo-CIDNP effect [1], was found in 1994 by Zysmilich and McDermott (ref JACS) by continuous illumination of frozen and quinone-blocked natural photosynthetic reaction centers under magic-angle spinning (MAS) NMR conditions. Later, the solid-state photo-CIDNP effect has also been demonstrated in the flavin protein phototropin LOV1-C57S [2]. Since then, the search for more systems able to show the effect has been started.

Aureochromes constitute a class of biological blue-light receptors that contain flavin mononucleotide (FMN) as chromophore in its light-oxygen-voltage (LOV) domain. FMN absorbs UV-A/blue light and undergoes a cyclic photoreaction which involves an intermediate flavin-cysteinyl adduct formation. The mutation in aureochrome-LOV (C287S) abolishes the adduct formation and shows a solid-state photo-CIDNP effect in MAS NMR. The mutation in the LOV domain leads to an increased lifetime of 3FMN and further induces a less competitive electron transfer from a distant tryptophan (Trp) to FMN. A tentative photo-cycle involving the generation of a spin-correlated radical pair [FMN•−–Trp•+] is proposed. Possible mechanisms that cause the solid-state photo-CIDNP effect are presently investigated by employing aureochrome LOV-C287S as a model system.

The solid-state photo-CIDNP experiment allows for investigation of both, the electronic ground-state and the radical pair state of the molecules forming a radical pair. The chemical shift of photo-CIDNP signals indicates the electronic environment of the ground-state structure after photo-cycle and the signal intensities can provide information of the electron spin density distribution on the induced radical pair at the atomic resolution.

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Similar properties of biosynthetic and catabolic 2-alkyl-4(1*H*)-quinolone 3-monooxygenase orthologs

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Keywords: group A flavin-dependent monooxygenases, *Pseudomonas aeruginosa*, membrane protein, aromatic hydroxylation

The opportunistic pathogen *Pseudomonas aeruginosa* uses the alkyl quinolones (AQ) HHQ (2-heptyl-4(1*H*)-quinolone (HHQ) and 2-heptyl-3-hydroxy-4(1*H*)-quinolone (PQS) as quorum sensing molecules. AQ signaling is integrated in a complex system of quorum sensing circuits which together regulate the expression of a wide range of genes, including many involved in virulence factor production and host colonization. Within the AQ biosynthetic pathway, HHQ hydroxylation to PQS is catalyzed by the flavin-dependent monooxygenase PqsH. Interestingly, a similar flavin monooxygenase, termed AqdB, was identified in the pathogen *Mycobacterium abscessus*. AqdB, together with a hydrolase and a dioxygenase, is part of a catabolic pathway mediating degradation of HHQ via PQS to anthranilic acid[1].

Both AqdB and PqsH belong to the group A flavin-dependent monooxygenases, which usually use NAD(P)H as electron donor and FAD as cofactor. Expression of *pqsH* or *aqdB* in the *P. aeruginosa* Δ*pqsH* mutant restored the ability to produce PQS. Analysis of the subcellular localization of the (MBP- or His-tagged) proteins when expressed in *Escherichia coli* suggested that both proteins are associated with the membrane, while cytoplasmic expression was inhibited by binding of the heat shock chaperone GroEL. Purified MBP-PqsH and MBP-AqdB proteins contained only small amounts of bound FAD, indicating weak FAD binding affinity. Truncation of an amphipathic C-terminal α-helix, which was assumed to contribute to membrane association, did not change the subcellular localization of recombinant PqsH or AqdB. Both proteins exhibited only low NADH-dependent oxygenase activity, which could be increased several-fold by the addition of *E. coli* cell free extract or detergent-solubilized *E. coli* membrane preparations. These observations suggest that both flavin-dependent monooxygenases may use electron donor(s) other than NAD(P)H.

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Newly designed and discovered monooxygenases for synthesis of ECL with enhanced stability and reduced inhibition

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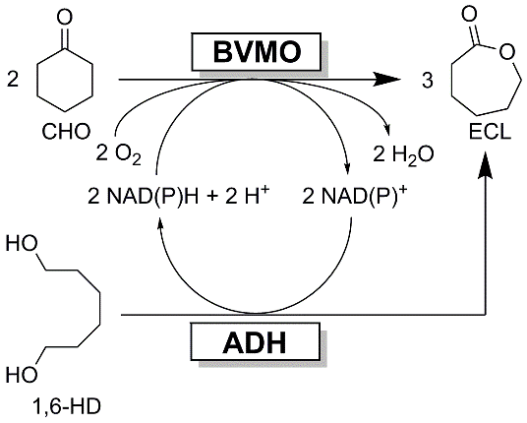
Keywords: Baeyer-Villiger monooxygenase, protein engineering, long-time stability, enzymatic cascade

Epsilon-caprolactone (ECL) is a key polymer precursor with a global annual production of multi-kilo tons.[1] The polycaprolactone (PCL), synthesised from ECL, is a biodegradable thermoplastic polymer with a wide range of applications in chemical industry.[1] Since the chemical synthesis of ECL and consequently PCL involves toxic and explosive chemicals,[1,2] developing an enzymatic synthesis pathway is an important issue. Enzymatic synthesis of ECL *via* a linear cascade approach starting from cyclohexanol (CHL) was developed[3,4,5] and extended[2]. Recently, a novel enzymatic convergent cascade approach was reported.[6,7] The convergent cascade utilises cyclohexanone (CHO) for the BVMO-catalysed oxidation and at the same time converts the second substrate 1,6-hexanediol (1,6-HD) by an alcohol dehydrogenase (ADH) to the final product ECL (Scheme 1). Regarding the Baeyer-Villiger oxidation of CHO, although cyclohexanone monooxygenase (CHMO) from *Acinetobacter* sp. NCIMB 9871 is considered as a model enzyme, limitations such as low operational stability, substrate- and product inhibition issues restrict its industrial application.

Scheme 1: *Synthesis of ECL via coupling of BVMO (Baeyer-Villiger monooxygenase) and ADH in a convergent cascade fashion.*

Therefore, we conducted a survey of different CHMO mutants in terms of higher long-term stability and reduced inhibition issues. The screening revealed two new CHMO variants (CHMO M15_L323C_A325C and CHMO M16_L323C_A325C) with increased long-term stability. Additionally, CHMO M15_L323C_A325C shows reduced substrate inhibition. Furthermore, the discovery of a putative BVMO from *Aspergillus flavus* NRRL3357 provides another promising candidate for the convergent cascade. The BVMO from *Aspergillus flavus* NRRL3357 displays a significantly higher tolerance to CHO and ECL than the CHMO wild type. Hence, providing a possible solution to the challenge of substrate and product inhibition.



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PqsL, an arylamine-hydroxylating monooxygenase involved in 2-heptyl-4-hydroxyquinoline-*N*-oxide biosynthesis

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Keywords: group A monooxygenase, amine hydroxylation, antibiotic, *Pseudomonas aeruginosa*

2-Alkyl-4-hydroxyquinoline *N*-oxides (AQNOs) produced by the opportunistic pathogen *Pseudomonas aeruginosa* are antibiotic compounds deriving from the alkyl quinolone (AQ) biosynthetic pathway, which also mediates the production of the signaling molecules 2-heptyl-4(1*H*)-quinolone (HHQ) and 2-heptyl-3-hydroxy-4(1*H*)-quinolone (PQS). However, despite extensive studies, the enzymatic synthesis of AQNOs has not been elucidated so far. We investigated the structure and function of the flavin-dependent monooxygenase PqsL, the key enzyme for AQNO formation. Although PqsL can be classified as group A flavin-dependent monooxygenase, it catalyzes the hydroxylation of a primary aryl amine rather than an aromatic ring. Its substrate, 2-aminobenzoylacetate (2-ABA), the central intermediate of the AQ biosynthetic pathway, is converted to the highly unstable 2-hydroxylaminobenzoylacetate (2-HABA). Intriguingly, both, 2-ABA and the PqsL product 2-HABA are substrates of the same downstream condensing enzyme. Therefore, the ratio between *N*-oxide and non-*N*-oxide products is influenced by a complex interplay of enzyme kinetic properties and decomposition processes.

Interestingly, PqsL shows some anomalies regarding co-substrate utilization as well. Refinement of available structural data and comparison to *p*-hydroxybenzoate hydroxylase, the model enzyme among group A flavin-dependent monooxygenases, revealed that structural features essential for NAD(P)H binding are missing in PqsL. Consistently, PqsL exclusively utilized reduced flavin as electron donor *in vitro*, which so far is unprecedented amongst group A monooxygenases. A deletion mutant of *P. aeruginosa* PAO1 lacking the *hpaC* gene, which codes for a flavin reductase involved in *p*-hydroxyphenylacetate degradation, showed an elevated AQ to AQNO ratio compared to the wild-type strain. Hence, it is likely that the availability of cytosolic free reduced flavin has influence on PqsL functionality and free flavin indeed is the enzyme's physiological electron donor.

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Two tyrosine residues, Tyr108 and Tyr503, are responsible for substrate activation in vanillyl alcohol oxidase

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Keywords: deprotonation, mechanism, tyrosine, vanillyl alcohol oxidase

Vanillyl alcohol oxidase (VAO) from *Penicillium simplicissimum* is a flavin-dependent oxidase that catalyzes the oxidation of *para*-substituted phenols. In order for oxidation to occur, the substrates must first be bound in the active site of VAO in their phenolate form. The crystal structure of VAO reveals that two tyrosine residues, Tyr108 and Tyr503, are positioned so as to facilitate the deprotonation of the substrates. Here, we created three VAO variants, Y108F, Y503F and Y108F/Y503F, and studied their biochemical properties. Kinetic studies revealed that the presence of at least one of the tyrosine residues is essential for efficient catalysis by VAO. Binding studies disclosed that the competitive inhibitor isoeugenol is predominantly in its deprotonated form when bound to wild-type VAO, but predominantly in its protonated form when bound to the variants. These results indicate that Tyr108 and Tyr503 are both involved in the activation of substrates in VAO, shedding light on the molecular mechanism of this flavin-dependent oxidase.

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Human apoptosis inducing factor: From its molecular mechanism to its functional and pathological significance

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Keywords: Apoptosis Inducing Factor, dimerization, NADH biding, partner interactions

The mitochondrial Apoptosis Inducing Factor (AIF) was firstly described as a caspase-independent death effector[1]. Upon an apoptotic stimulus, AIF is released from the mitochondrial intermembrane space, then translocating to the nucleus, where induces large-scale DNA fragmentation. Beyond its apoptotic function, AIF is a ubiquitous eukaryotic flavoprotein that is key for cell survival and for normal development. The NADH oxidoreductase activity of AIF was reported to be essential in the mitochondrial bioenergetics by indirect regulation of the biogenesis of major respiratory chain complexes[2]. In humans, mutations in AIF cause severe neurological disorders associated to OXPHOS failure and increase of its cytocidal potential[3,4]. Moreover, NADH binding modulates AIF monomer-dimer equilibrium in healthy mitochondria and could be determinant in its biological interactions with other molecules including a variety of protein partners[5]. Further characterization of the AIF molecular activity is key for a better understanding of the mechanism of the diseases in which it is involved and for exploring new therapeutic approaches. In this context, we are using a multidisciplinary methodology combining biochemical, biophysical and cellular techniques to understand the AIF physiological role in cellular death and life, as well as to identify compounds that might modulate these activities[5,6].

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Human FMO5: discovery of Baeyer-Villiger reactions in human oxidative metabolism

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Keywords: human drug metabolism, Baeyer-Villiger oxidation, membrane-associated enzyme, biocatalysis.

Human flavin-containing monooxygenases (hFMOs) are membrane-associated enzymes which play a key role in detoxification of xenobiotics and bioactivation of drugs[1]. The common feature of these nicotinamide-dependent proteins is the use of molecular oxygen to oxidize compounds containing nucleophilic heteroatoms. While FMO5 shows the most abundant expression in the liver among the five human isoforms, its functional activity has not yet been clearly established and classical FMO substrates are poorly converted or non-reactive.

In an effort to characterize its oxidative catalytic activity as well as to elucidate its 'obscure' substrate tolerance, hFMO5 was first studied towards NADPH and oxygen via analysis of the spectral behaviour of its FAD cofactor. A comprehensive substrate profiling was then carried out, thereby confirming the poor activity towards typical FMO substrates. Most importantly, hFMO5 could be identified as a Baeyer-Villiger monooxygenase (BVMO) displaying a rather large tolerance on model substrates. Aliphatic and cyclic ketones could be converted to the corresponding esters or lactones, respectively. Aldehydes were accepted as well, whereby regio-divergent oxidation yielded either the corresponding carboxylic acid or formate ester as product. The regio- and stereoselectivity pattern of hFMO5 was fully characterized and compared to that of classical BVMO enzymes[2].

In line with this and reflecting the significance of a BV activity in human drug metabolism, two approved drug molecules bearing the carbonyl moiety on a long aliphatic chain were identified as substrates of FMO5. These findings open new perspectives in human oxidative metabolism.

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L- and D-6-hydroxynicotine oxidase catalyze carbon-nitrogen bond oxidation, not carbon-carbon bond oxidation

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Keywords: amine oxidation, hydroxynicotine oxidase, mechanism, site-directed mutagenesis

The flavoproteins L-6-hydroxynicotine oxidase (LHNO) and D-6-hydroxynicotine oxidase (DHNO) catalyze complementary reactions in the oxidation of nicotine. LHNO is a member of the monoamine oxidase structural family, while DHNO is a member of the vanillyl oxidase structural family. Both enzymes have long been assumed to catalyze oxidation of a carbon-carbon bond in their respective substrates, forming 6-hydroxypseudooxynicotine. We have used mass spectrometry and NMR spectroscopy to show that these enzymes instead catalyze oxidation of a carbon-nitrogen bond in their substrates. Site-directed mutagenesis and pH effects have been used to propose new mechanisms for both enzymes that are consistent with this observation.

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PockeMO: a polycyclic ketone monooxygenase as the first 3D structure from the BVMO subclass active on bulky substrates

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Keywords: Baeyer-Villiger monooxygenase, thermostability, steroids, X-ray crystallography

Baeyer-Villiger monooxygenases are well-established enzymes for biocatalysis, transforming ketones to esters or lactones via a reactive peroxyflavin. In contrast to the chemical route, catalysis is performed under benign conditions, and both the co-substrate – molecular oxygen – and the only byproduct – water – can be considered as "green" as it gets. Furthermore, many enzymes are promiscuous in their substrate acceptance and can perform transformations regio- and stereoselective. However, there are two major limitations for industrial application: a lack of enzymes active on large, functionalized molecules as is the case for many drugs; and enzyme variants that can tolerate heat and organic solvents.

We present here the discovery and characterization of a BVMO from the fungus *Thermothelomyces thermophila* that addresses both issues: the recombinant enzyme is more thermostable than most BVMOs described to date, and is additionally remarkably active on bulky compounds. The enzyme can be attributed to a subclass of BVMOs that are known for this type of substrate scope, and by solving the crystal structure we are now able to link their common structural features to this activity. By performing conversions with a large variety of substrates we determined a high substrate promiscuity and a particular preference for polycyclic ketones, hence we named the enzyme PockeMO. Most notably, we could convert several steroid substrates with excellent yields, and found that PockeMO is the first BVMO able to oxidize on the A and D ring, as well as on the C17 sidechain.

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Inhibition of 'thermophilic-like' ene-reductases by an *N*-terminal cysteine

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Keywords: old yellow enzyme, flavoenzyme, ene-reductase, mutagenesis

Old Yellow Enzymes (OYEs) belong to the class of flavoenzymes and reduce various α,β-unsaturated substrates, producing chiral molecules. This makes them potentially interesting for biocatalysis. An *N*-terminal cysteine residue, occurring only in thermophilic-like OYEs, was found to function in the binding of the flavin cofactor and therefore modulates the flavin reduction potential.[1] Herein, we characterize the thermophilic-like OYE*R*02a from *Rhodococcus opacus* 1CP[2] as well as its engineered variants C25A, C25S and C25G. We show, that the substrate maleimide drastically decreases the specific activity of the wild type, due to a Michael addition forming a thioether. However, the cysteine-lacking variants C25S and C25G are not inhibited, showing high specific activities on *N*-methylmaleimide (109 U mg^{-1}) using NADPH as a cofactor. Interestingly, the choice of cofactor seems to play a major role in the reduction of the substrate although OYEs are known to operate in a bi-bi ping-pong mechanism.[3] More precisely, with the mimic cofactor BNAH[4] and maleimide as a substrate no substrate inhibition was observed and the specific activity was highest for the wild type. Using 2-cyclohexen-1-one as a substrate (and NADPH), the specific activities ranged from 3.7 U mg^{-1} for the wildtype to 0.3 U mg^{-1} for the C25G variant. In addition, the catalytic efficiencies were much lower for 2-cyclohexen-1-one than for *N*-methylmaleimide. To conclude, we show that the combination of site-directed mutagenesis and cofactor optimization provides access to more robust OYEs.

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Over-expression in *Escherichia coli* and purification of a novel isoform of human FAD synthase

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Keywords: Flavin, over-production, FAD synthase, *E. coli*.

Flavin adenine dinucleotide (FAD) is a redox cofactor essential for many enzymatic reactions in humans. It is synthesized from dietary derived riboflavin through a couple of reactions catalyzed by Rf kinase (EC 2.7.1.26) and FAD synthase (EC 2.7.7.2). To date different human FAD synthase transcripts deriving from alternative splicing of FLAD1 gene, have been deposited in databases. We demonstrated that longest transcript codes for isoform 1 which has mitochondrial localization, whereas the isoform 2 has cytosolic localization 1. We characterized isoform 1 and 2 of the human FAD synthase, in some details and we demonstrated that it is a bi-functional enzyme characterized by a C-terminus PAPS reductase domain involved in FAD synthesis and a N-terminus molybdopterin binding domain performing a FAD hydrolase activity 2. Recently mutations in FLAD1 gene have been described, leading to the discovery of a novel transcript, coding for a shorter isoform (namely isoform 6) consisting of the sole PAPs reductase domain 3. Here we confirmed the existence of this novel transcript in MegaMan human transcriptome library; therefore we amplified and cloned the corresponding cDNA in an *E. coli* expression vector inserting a 6His tag at the N-terminus of the protein. Several *E. coli* strains were transformed with the recombinant construct and screened for expression. Rosetta(DE3)pLysS revealed the most suitable. Cells were cultured at various temperatures and induction times. Best expression was obtained at 28 °C and 2 hours of growth after addition of IPTG 0.4 mM. The over expressed protein, exhibiting an apparent molecular mass of 34 kDa on SDS-PAGE, was collected in the soluble cell fraction and was purified to homogeneity by Nickel chelating affinity chromatography. A final yield of 7 mg protein/L of cell culture was obtained, pure enough to perform functional characterization (P-Leone poster at this meeting).

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Molecular mechanism of TMAU-causing N61S variant and structural function of conserved Asn61 in hFMO3

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Keywords: monooxygenase, trimethylaminuria, binding affinity, C4a-hydroperoxyFAD intermediate.

Human flavin-containing monooxygenase 3 (hFMO3) participates in the monooxygenation of drugs and other xenobiotics. Some hFMO3 allelic variants results in decreased or abolished functional activity for trimethylamine (TMA) N-oxygenation thus leading to the trimethylaminuria (TMAU or fish-odor syndrome). Although several TMAU-causing variants have been identified in patients and well characterized in vitro, the underlying molecular mechanism for TMAU is still elusive. Here, the loss-of-function N61S variant is used as a model to investigate its inactivation mechanism. Thermodynamic (ITC, DSC), spectroscopic (CD) and transient kinetics (Stopped-flow) experiments reveal that the poor NADP(H) binding affinity caused by N61S mutation results in the unstable C4a-hydroperooxyFAD intermediate and makes the enzyme quite inefficient in using NADPH for flavin reduction. In addition, structural function of the highly conserved Asn61 is also investigated by an integrated experimental (ITC, DSC, CD, site-directed mutagenesis, steady-state and pre-steady-state kinetics) as well as computational (in silico mutagenesis and molecular docking) procedure. The data demonstrate that steric hindrance and/or the properties of the residue itself lead to the poor NADP(H) binding affinity, suggesting the conserved Asn61 residue helps in the correct positioning of the NADP(H). This work adds insight into the molecular mechanism of fish-odour syndrome and structural function of highly conserved Asn61 residue in hFMO3.

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Trapping SMO in an FAD-exchange configuration

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Keywords: styrene monooxygenase, two-component flavoprotein

Styrene monooxygenase (SMO) from *P. putida* (S12) is a two-component flavoprotein composed of NADH-specific flavin reductase (SMOB) and FAD-specific, styrene epoxidase (SMOA). Catalysis by SMO involves the reduction and transfer of FAD from SMOB to SMOA followed by enantioselective epoxidation of styrene by SMOA. Although many features of the catalytic mechanism SMO have been reported, the structure of transient SMOA-SMOB complex thought to be involved in FAD-exchange has remained elusive [1]. In the apo-protein crystal structure of SMOA (PDB id: 3IHM) FAD-binding sites are sequestered in a resting state inaccessible to SMOB. The closely related structure of 4-hydroxybenzoate-3-hydroxylase (PDB id: 1PBE) reveals an alternative subunit interface (FAD-exchange state) that could provide SMOB direct access to the active site of SMOA [2]. We propose that in catalysis the binding energy of reduced FAD is coupled to the conversion of SMOA from apo-resting state to an FAD-exchange state.

To evaluate this hypothesis of alternate subunit interfaces in catalysis, structure files of SMOA subunits were submitted to the ClusPro 2.0 protein interactions server [3]. Inspection of the resulting models indicate that lysine residues sequestered in the interface of the resting-state subunits are exposed in the exchange-state. After anaerobic labeling of SMOA(FADred) with the lysine reactive probe, Dansyl-Cl, followed by FAD-reoxidation, the enzyme retains activity, but binds tightly to FADox. This feature is consistent with the exchange-state of SMOA, which is postulated to bind FAD with high affinity relative to the resting state. By this procedure we have disrupted the resting-state interface of SMOA and trapped the enzyme in an active configuration related to the FAD-exchange state.

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Novel Baeyer-Villiger monooxygenases discovered by genome mining in a *Streptomyces* from the Atacama Desert

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Keywords: Baeyer-Villiger monooxygenases, genome mining, extremophiles

Baeyer-Villiger monooxygenases (BVMOs) are a prominent example of oxidative enzymes, which catalyze not only Baeyer-Villiger reactions, but also halogenations, epoxidations and sulfoxidations with high chemo-, region- and stereoselectivity. Among BVMOs, the NADPH-dependent Type I BVMOs have been the most studied. Also, a novel group of BVMOs that display relaxed coenzyme specificity, by accepting both nicotinamide cofactors, was recently described and named type II FMOs. Type I BVMO and Type II FMOs are of great potential for biocatalytic industrial applications. In order to study this enzymes, we search in the proteome of *Streptomyces leeuwenhoekii*, an Actinobacteria isolated from the Atacama Desert, the driest desert in the world. Genome mining using previously described typifying motifs[1,2] revealed three putative Type I BVMOs and two putative Type II FMOs. A phylogenetic tree was generated including representative enzymes from both subclases and the five BVMOs from *S. leeuwenhoekii*. Two of the Type I BVMO are clustered with CPDMO and CDMO (described for cyclic ketones acceptance), two with the Type II FMOs cluster and the last one in a cluster related to HAPMO (described for aromatic substrate). The genomic DNA from *S. leeuwenhoekii* was used as template to amplify the 5 putative BVMO sequences. Genes were cloned using a ligation free method and heterogously expressed as soluble proteins using the pCRE2/*E. coli*NEB10β expression system. Cofactor and substrate specificities were studied for each of the proteins by using NAD(P)H depletion assay. Next steps include the study of the novel enzymes in terms substrate enantioselectivity, kinetics parameters, and protein structure.

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The ins and outs of vanillyl alcohol oxidase: Identification of ligand migration paths

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Keywords: ligand diffusion, flavoprotein, oxygen reactivity, Protein Energy Landscape Exploration (PELE)

Vanillyl alcohol oxidase (VAO) from *Penicillium simplicissimum* is a homooctameric flavoenzyme with a covalently bound FAD cofactor. The VAO-dimer is the smallest functional unit, and each subunit contains two domains, the FAD-binding and the cap-domain. Amongst the many reactions VAO catalyses is the two-step conversion of p-creosol (4-methylphenol) to vanillin (4-hydroxy-3-methoxybenzaldehyde).

We have performed Monte Carlo based simulations on dimeric VAO to understand how the substrates and products of the abovementioned reaction enter and exit the active site. For the phenolic ligands, one entry/exit path via the subunit interface leading to the *si*-side of the flavin, as well as two additional exit paths were identified. For the co-ligands dioxygen and hydrogen peroxide, a fourth path at the interface of the two domains of the VAO subunit, leading to the *re*-side of flavin, was discovered.

We propose a sequence of events leading substrates to the active site and products away from the active site. We also propose two concierge residues, which grant access to the active site for phenolic ligands as well as two gateway residues involved in co-ligand migration to the active site.

This study presents for the first time a comprehensive overview of ligand and co-ligand migration in a member of the VAO/PCMH family.

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Real-time analysis of conformational control in electron transfer reactions of diflavin oxidoreductases.

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Keywords: Electron transfer, FRET, Protein dynamics, diflavin oxidoreductase

Biological electron transfer and protein domain dynamics are often associated. However, as it is experimentally challenging to trap catalytically relevant conformational sub-states, little is known about the role of protein domain dynamics during enzyme catalysis. For the diflavin oxidoreductases family, the importance of dynamics has been inferred from a variety of structural, single-molecule, and ensemble spectroscopic approaches. However, no experimental methods have been able correlate diflavin oxidoreductase motions with chemical steps during turnover. To address this shortcoming, we have labelled two physiologically important diflavin oxidoreductases, cytochrome P450 reductase and neuronal nitric oxide synthase (nNOS), with donor and acceptor fluorophores. By using both UV-Vis and FRET stopped-flow approaches, we have been able to detect transient chemical and conformational states that occur during diflavin oxidoreductase catalysis. Moreover, by making use of 5-deazaflavin mononucleotide (as a thermodynamic block) and isotopically labelled nicotinamide coenzymes, we have correlated the timing of diflavin oxidoreductases structural changes with key mechanistic steps (coenzyme binding/reaction chemistry).

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Enantioselectivtiy of styrene monooxygenases towards sulfides is defined by a tyrosine in the active site

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Keywords: styrene monooyxgenase, enantioselectivity, sulfoxidation, phylogeny.

Styrene monooxygenases (SMOs) are flavoproteins consisting of two-components, the monooxygenase part (StyA) and the reductase part (StyB) [1]. The latter one delivers reduced FAD to StyA, which is used to activate molecular oxygen for the reaction with an aromatic substrate. SMOs can be distinguished into E1-type enzymes, which are usually part of a styrene degradation cluster and an E2-type, that is not part of a certain cluster, but supposed to be involved in the detoxification of indole. The E2-type includes an oxygenase StyA1 and a construct StyA2B representing a natural fusion of StyA and StyB into a single protein [2]. SMOs catalyze the regio- and enantioselective epoxidation of styrene to (*S*)-styrene oxide but also indole oxygenation and selective sulfoxidations, which are of interest for industrial application. However, the preference for an enantiomer can differ.

To rationalize this finding, a phylogenetic analysis of known and putative SMOs was conducted. 25 candidates from different branches were selected, cloned, expressed and screened for indigo formation. 7 of them showed no color formation in this test, indicating inactivity towards indole and/or malfolding. The remaining 18 SMOs were synthesized, purified and characterized according to activity, substrate spectrum and enantioselectivity.

Results obtained clearly show that the activity differs randomly among the groups. Interestingly, in most cases the sulfoxidation activity towards thioanisole and benzyl methyl sulfide is higher compared to styrene. Further, there is a clear prevalence towards the (*R*)-enantiomer for the E1-type and the (*S*)-enantiomer for E2-type SMOs in sulfide conversions. Lin et al. showed by mutation that a tyrosine in the active site changes the enantioselectivity for 1-phenylcyclohexanone [3]. Sequence analysis as well as structural modelling indicate that this amino acid change also reflects the enantioselectivity towards sulfides of E1- and E2-type SMOs and that Tyr73 acts as the guiding residue for substrate binding.

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Unexpected influence of NH_4^+ on the photocycle of aureochrome 1a LOV domain from *Phaeodactylum tricornutum*

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Keywords: FMN (flavin mononucleotide), LOV (light-oxygen-voltage) domain, photocycle, crystallography, UV-Vis spectroscopy.

Aureochromes are blue-light receptors, containing a unique fusion between a LOV domain (sensor) and a DNA-binding basic leucine zipper domain (effector).1 The blue light sensor is a flavoprotein, since it is binding FMN as a cofactor. In the dark state, the FMN cofactor is non-covalently bound, but upon blue-light illumination a covalent link between cysteine 287 and the C4-atom of the isoalloxazine ring is formed.2 This lit state was shown to be the functional state of the aureochromes. This adduct formation triggers structural changes, which involves firstly an unfolding of the flanking helices (A´α- and Jα-helix) of the LOV domain and secondly the dimerization of the LOV domain.3 Using UV-Vis spectroscopy, adduct-formation can be followed by a shift from 450 nm (free Flavin) to 390 nm (C4-adduct).2, 3

Based on the dark state structure of the aureochrome 1a LOV domain from *Phaeodactylum tricornutum* (*Pt*Au1aLOV)2, an engineered light-sensitive mutant (*Pt*Au1aLOVV349W) shows a distantly altered interface. Interestingly, in *Pt*Au1aLOVV349W the cofactor FMN seems to be permanently covalent bound according to UV-Vis spectroscopy. However, the crystal structure at a resolution of 2.15 Å shows a non-covalent bound flavin. This observation was confirmed by *in crystallo* spectroscopy. Titration experiments revealed that the ammonium ion (NH_4+), which was present in the crystallization conditions, has an influence on the photocycle of *Pt*Au1aLOVV349W. NH_4+ induces the decay of the C4-adduct and initiates a spectral shift from 390 nm to 450 nm in the light excited state. These results demonstrate the influence of cations towards the photocycle of LOV domains and lead to new strategies of engineering mutants with distinct photochemical behavior.

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Activation of the class Ib ribonucleotide reductase by a flavin network in *Bacillus cereus*

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Keywords: enzyme activation, flavodoxin reductase, ribonucleotide reductase, structure.

Several enzyme systems are activated through flavodoxin / flavodoxin reductase pathways. For instance, in the class Ib ribonucleotide reductases (RNRs) the flavodoxin-like protein NrdI is essential in one of the redox networks that together activate the RNR enzyme system. RNRs reduce ribonucleotides to deoxyribonucleotides by employing radical chemistry, and NrdI activates the dimanganese center in the radical generating RNR β subunit NrdF. As seen by crystallography, the NrdI protein binds to NrdF, generating a gated channel between the FMN site in NrdI and the dimanganese center in NrdF, which allows for transfer of superoxide and activation of the RNR class Ib.[1]

It has been proposed that NrdI itself is recycled *in vivo* by an NrdI reductase, but no NrdI reductase has been identified. Flavodoxin-NADP+ oxidoreductases (FNRs) are probable reductants of NrdI. We identified three, thioredoxin-like flavodoxin reductases in the genome of *Bacillus cereus* and carried out structural and functional studies in order to characterise their ability to reduce NrdI. Binding studies showed that all three FNRs bind NrdI with similar affinities, however, steady-state kinetics revealed that one FNR reduces NrdI at a much higher rate than the other two FNRs.[2,3] Using this FNR as an NrdI reductase, we were also able to activate the NrdF under aerobic conditions, mimicking cellular conditions. Altogether, our observations suggest that this FNR might be the superior NrdI reductase *in vivo*, thereby completing the flavin activation network of class Ib RNR.[2] The activation and interaction between NrdI and FNR is likely linked to the observed rotation of the NADPH domains relative to the FAD domains in the *Bacillus cereus* FNR crystal structures.

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Development of a FACS-based screening for H_2O_2 producing oxidoreductases using GFP derived sensors

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Keywords: high-throughput-screening, H_2O_2, roGFP2-Orp1, FACS

Fast and robust screening platforms represent a vital prerequisite for the engineering of any enzyme class in research and industry. Current screening methods for oxidoreductases often rely on colorimetric detection of diffusible electron acceptors or the products of secondary reactions, making artificial compartmentalization in, e.g. microtiter plates necessary. Consequently, this requires large amounts of material, time and manual handling and restricts efforts to screen large libraries with desirable speed.

In this work, we adopt the green fluorescent protein (GFP) fusion construct roGFP2-Orp1 as a genetically encoded H_2O_2 sensor. Its consumption of hydrogen peroxide results in a stoichiometric fluorescent output and makes it well-suited for assessing the enzymatic turnover of H_2O_2 producing enzymes *in vitro* and *in vivo*. (1)

In a first assessment, we could confirm the interaction of hydrogen peroxide in nanomolar concentrations and purified roGFP2-Orp1 *in vitro*. We successfully used the fluorescent reporter in combination with a collection of *Trametes multicolor* pyranose-2-oxidase (*Tm*P2O) variants. In addition, the sensor was effectively employed to elucidate substrate preferences of *Tm*P2O. In order to employ this fluorescent sensor for whole cell screening of enzyme activity with FACS, we co-express roGFP2-Orp1 and enzyme(s) of interest. The redox sensor will be immobilized on the surface of yeast cells, and can be combined with cytoplasmic expression and surface display of peroxide generating enzymes.

We predict, that an advanced high-throughput screening based on fluorescent activated cell sorting (FACS) will allow exploring a notably higher degree of mutational space in enzyme libraries. Comprehensive studies on oxygen reactivity or substrate preference of certain flavoenzymes will greatly benefit from it and the identification of improved biological catalysts for industrial applications will be simplified.

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Reduction of CO_2 by the molybdenum- and flavin-containing formate dehydrogenase from *Cupriavidus necator*

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Keywords: provide four keywords separated by commas.

The ability of the FdsABG formate dehydrogenase from *Cupriavidus necator* (formerly known as *Ralstonia eutropha*) to catalyze the reverse of the physiological reaction, the reduction of CO_2 to formate utilizng NADH as electron donor, has been investigated. Contrary to previous studies of this enzyme, we demonstrate that it is in fact effective in catalyzing the reverse reaction, with a k_{cat} of 10 s^{-1}. We also quantify the stoichiometric accumulation of formic acid as the product of the reaction and demonstrate that the observed kinetic parameters for catalysis in the forward and reverse reaction are thermodynamically consistent and comply with the expected Haldane relationships. Finally, we domonstrate the reaction conditions necessary for guaging the ability of a given formate dehydrogenase or other CO_2-utilizing enzyme to catalyze the reverse direction so as to avoid false negative results. In conjunction with our earlier studies on the reaction mechanism of this enzyme[1], on the basis of the present work we conclude that all molybdenum- and tungsten-containing formate dehydrogenases likely operate via a simple hydride transfer mechanism and are effective in catalysing the reversible interconversion of CO_2 and formate.

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Light-driven enzymatic regeneration of the NADH cofactor using a flavoenzyme

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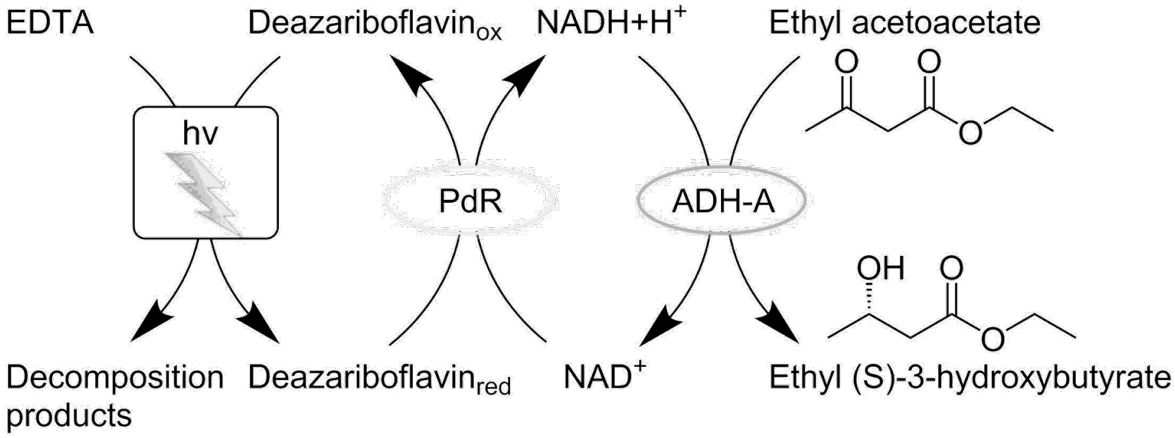
Keywords: NADH regeneration, deazariboflavin, photo-chemo-enzymatic cascade



Scheme 2. The proposed photo-chemo-enzymatic cascade

Oxidoreductases catalyse highly selective redox reactions. To supply the redox equivalents necessary for the reaction, oxidoreductases require cofactors, for instance NAD(P)H. The high cost for these cofactors make their application in catalytic amounts together with *in situ* regeneration of the catalytically active form inevitable. Next to well-established enzymatic methods, also photo-enzymatic methods may be interesting alternatives [1,2]. Light can excite a photosensitizer to a higher energy level, enabling reactions otherwise impossible. The photosensitizer is reduced while a sacrificial electron donor is consumed. Subsequently, the reduced photosensitizer reduces a catalyst, in this case the flavoenzyme putidaredoxin reductase (PdR). The PdR reduces the cofactor NAD$^+$ to NADH. Furthermore, the regeneration system is coupled to a redox reaction catalysed by the alcohol dehydrogenase from *Rhodococcus ruber* DSM 44541 (ADH-A)[3]. The scope and the limitations of this approach will be discussed.

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Cupriavidus necator JMP134 encodes for two active *p*-hydroxybenzoate hydroxylases with different properties

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Keywords: selective oxygenation, dye production, extinction coefficient, indigo

4-Hydroxybenzoate is initially converted to 3,4-dihydroxybenzoate during the microbial degradation (peripheral route). The latter is a key metabolite in the metabolism of aromatic compounds and funnels into the central pathways. The hydroxylation reaction is catalyzed by a flavin dependent hydroxylase designated as *p*-hydroxybenzoate hydroxylase (PHBH; EC 1.14.13.2) [1,2]. This enzyme class is extensively studied for biochemical, structural as well as applicative reasons. An issue still enigmatic is the dependency of the nicotinamide cofactor (NADH vs. NADPH) for FAD reduction which allows subsequently activation of molecular oxygen and thus substrate hydroxylation.

A well-known degrader of aromatic compounds is *Cupriavidus necator* JMP134 [3] and this strain encodes for two PHBHs (*pobA1* and *pobA2*). Respective genes were identified by genome mining and ordered as codon optimized variants for cloning. Constructs and gene expression were successfully achieved. Both proteins were obtained as His_{10}-tagged variants and purified to homogeneity. Protein preparations were used to characterize the enzymes for the dependency on nicotinamide cofactors as well as for product formation. The enzymes showed a similar activity (*Cn*PHBH1: 6.10 to 9.97 U mg-1 and *Cn*PHBH2 23.62 to 39.95 U mg-1) whereas only *Cn*PHBH1 accepted both cofactors. *Cn*PHBH2 was strictly dependent on NADPH. This result will be discussed by means of a phylogenetic study. Further, strain *Cupriavidus necator* JMP134 was cultivated with 4-hydroxybenzoate as carbon source and crude extract obtained of biomass was assayed for PHBH-activity and this is discussed in relation to the kinetic data obtained from the recombinant enzymes.

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Nicotinamide Adenine Dinucleotide (NADH)-Dependent Convergent Cascade for Lactonizations

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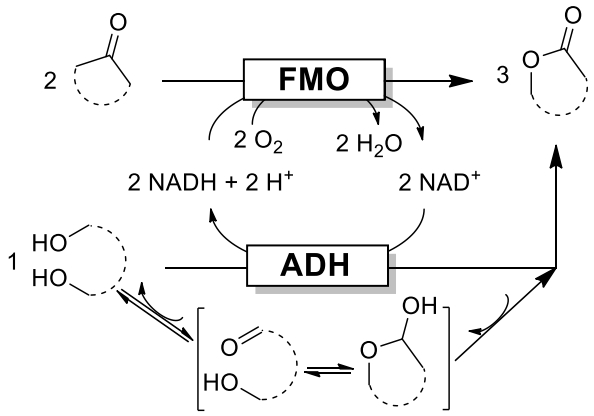
Keywords: Redox-Neutral Cascade, Nicotinamide Cofactor, Baeyer-Villiger Monooxygenase, Alcohol Dehydrogenase

Multi-enzymatic cascade reactions running in one-pot without isolation of intermediates offer considerable advantages: time and cost needed for the recovery of product is reduced, reversible reactions can be driven to completion and the harmful or unstable compounds can be kept at the minimum level.[1] Redox enzymes that usually require expensive nicotinamide cofactors (NADH or NADPH) are mostly coupled in a cascade to make the reaction systems more efficient and economical. Currently, a new redox-neutral multi-enzymatic system was reported for the synthesis of ε-caprolactone in a convergent cascade fashion. In this system, a cyclohexanone monooxygenase (CHMO) catalyzes the oxidation of cyclohexanone and an alcohol dehydrogenase (ADH) catalyzes the double oxidation of 1,6-hexanediol for the *in situ* regeneration of the NADPH cofactor.[2] This system has been limited to NADPH due to the strict cofactor recognition of the CHMO from *Acinetobacter sp.* NCIMB 9871. From an industrial perspective, NADH is the preferred cofactor as it is cheaper (up to 30 times) and more stable than NADPH.[3]



Scheme 1. Convergent cascade reactions coupling the flavin-containing monooxygenase (FMO) with an alcohol dehydrogenase (ADH) for lactonizations. Two molar equivalents of ketone are coupled with one molar equivalent of diol to synthesize three molar equivalents of lactone.

Recently, some of us have identified a new class of flavoprotein monooxygenases, type II flavin-containing monooxygenases (FMOs).[4] With FMO-E, one of these new type II FMOs, and the well-studied horse liver alcohol dehydrogenase (HLADH), we have established now a new NADH-dependent redox-neutral convergent cascade (Scheme 1). Two model reaction cascades have been established for the synthesis of γ-butyrolactone and bicyclic lactones.

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Photoenzymatic regeneration of ene reductases using flavins as photocatalysts

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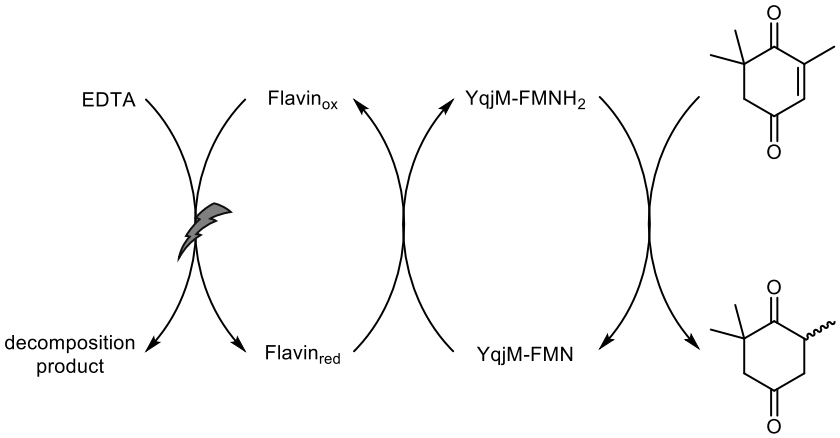
Keywords: alkene reduction, cofactor regeneration, old yellow enzymes, photoenzymatic reduction

Ene reductases, or Old Yellow Enzymes (OYEs), catalyse the reduction of C=C double bonds of α,β-unsaturated aldehydes and ketones utilizing reduced FMN as cofactor and NAD(P)H as a source of reducing equivalents. For biocatalytic applications, it is desirable to circumvent the use of NAD(P)H, since it is expensive and unstable, and requires an enzymatic regeneration system. One of the possibilities to overcome NAD(P)H regeneration is to use a light-driven reduction system. For this, EDTA is utilized as a sacrificial electron donor, providing reducing equivalents for photocatalytic oxidation via FMN as an externally added photocatalyst[1]. Blue LED light can efficiently promote this photocatalytic reaction[2]. For YqjM, a member of the OYE family isolated from *Bacillus subtilus*, photoenzymatic reduction of C=C bonds has been proven to be successful[1]. We are now exploring the use of these photoenzymatic reactions in more detail.



Light-driven regeneration of YqjM using flavins as photocatalysts.

As a model substrate, ketoisophorone is depicted.

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Role of redox potential in control of two-step reduction

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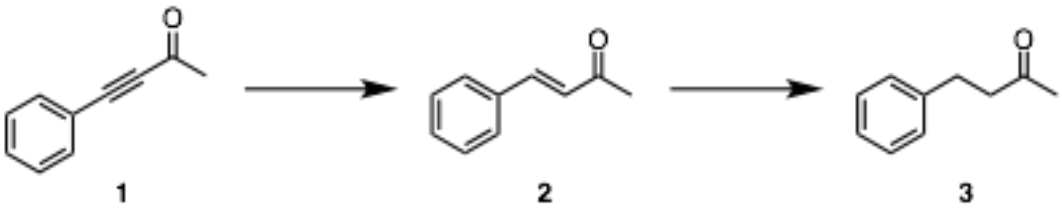
Keywords: biocatalysis, protein engineering, cofactor analogs, alkyne reduction



FMN-dependent Old Yellow Enzyme 1 (OYE1) from *Saccharomyces pastorianus* is a well characterized ene-reductase with activity towards α,β-unsaturated ketones. OYE1 has shown novel activity towards **1**, a phenyl-substituted alkyone.1 The two-step reduction of **1** to **3** by OYE1 proceeds with similar rates. In contrast, the engineered OYE variant cpOYE303 shows dissimilar rates for the two consecutive reactions.2 These rate discrepancies could be linked to changes in active site accessibility and lower FMN redox potential in the circular permutated OYE variant, revealing possible mechanistic difference for the two reduction steps.

To more thoroughly investigate this findings, we explored the functional impact of circular permutation on reduction of **1** in two OYE1 homologues; 12-oxophytodienoate reductase 1 and 3 (OPR1 and OPR3) from *Lycopersicon esculentum*. In addition, we have studied the effects of synthetic FMN analogs with altered redox potentials on the two-step conversion of 1 to 3.

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NMR and stopped-flow studies pinpoint key residues involved in enzymatic hydride transfer in PETNR flavoenzyme

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Keywords: hydride transfer, kinetic isotope effects, NMR, pentaerythritol tetranitrate reductase

Pentaerythritol tetranitrate reductase (PETNR) is a flavoenzyme that is able to reduce a wide variety of α,β-unsaturated compounds. As well as having high potential to be used in biocatalytic processes, PETNR is an excellent model system for studying H-transfer reactions. Mechanistic studies performed using stopped-flow methods have shown that tunneling contributes towards PETNR-catalyzed hydride transfer from the NAD(P)H coenzyme to the flavin cofactor and that fast protein motions might facilitate this catalytic step1,2. However, the difference in reactivity of the enzyme towards NADPH and NADH is still not clearly understood and the role of protein dynamics contributing in catalytic events requires an atomistic description of the enzyme. Herein, we employed a large variety of spectroscopic techniques in order to gain more insight into how the enzyme facilitates the H-transfer reaction. Using single-point mutagenesis coupled with stopped-flow methods, we identified key active-site residues involved in binding and/or hydride transfer and also how shortening the side-chains of more distal residues impacts the catalytic rate of the enzyme. For an atomistic description of the enzyme, we employed a nuclear magnetic resonance approach, thus characterizing sub-atomic fluctuations in the enzyme, using $NADPH_4$ an $NADH_4$ as ground state mimics. NMR binding studies coupled with the use of variable pressure and 15N backbone relaxation data enabled us to pinpoint catalytically relevant conformations and to characterize the frequency of dynamical regions in the enzyme.

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High resolution studies of hydride transfer in the ferredoxin-NADP+ reductase superfamily

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Keywords: hydride transfer, crystallography, active site compression, NADPH oxidase

The flavoenzyme ferredoxin-NADP+ reductase (FNR) catalyzes the transfer of electrons from photoreduced ferredoxin to NADP+ during photosynthesis and serves as a model for a broad superfamily of enzymes including NO synthase, cytochrome P450 reductase, and NADPH oxidases. Our goal is to define the mechanistic details of hydride transfer between FAD and NADPH using corn root FNR as a model.

In initial studies of spinach FNR crystals, the nicotinamide binding site was seen to be blocked by an aromatic side chain (Tyr) lying close and parallel to the *re*-face of the flavin. Using a Tyr to Ser mutant of pea FNR, 1.8 Å resolution structures with NADP(H) bound were obtained,[1] revealing an unexpected binding mode in which the nicotinamide ring laid against the FAD isoalloxazine at a ~30° angle. Although similar complexes have been seen in other FNR superfamily members, based on stopped-flow studies it has been claimed that these complexes are non-productive.[2] To resolve this question and better define the mechanistic details of hydride transfer, we carried out spectroscopic studies and determined higher resolution structures of FNR-NADP(H) complexes.

Here, we present the structures of wild type corn root FNR at ~1 Å resolution, along with ~1.5 Å resolution Y316A and Y316S mutants in complex with nicotinamide, NADP+, and NADPH. These enzymes are active and spectra of the crystalline complexes match those from solution studies. Also a reinterpretation of the earlier stopped-flow studies supports the relevance of these complexes to catalysis. Furthermore, our structures reveal more detailed information about the hydride transfer reaction. In particular, the complexes show higher anisotropic mobility of the C4 atom of NADP+ compared to NADPH, very short contact distances between NADPH and FAD, and distortion of FAD geometry that implicate active site compression as a key factor enhancing hydride transfer in the FNR superfamily.

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The interaction of human dimethylglycine dehydrogenase with electron transferring flavoprotein

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Keywords: Human electron transferring flavoprotein (hETF), dimethylglycine dehydrogenase (DMGDH), sarcosine dehydrogenase (SARDH), isobutyryl-CoA (IBD), very long-chain acyl-CoA dehydrogenase (VLCAD).

The human electron transferring flavoprotein (hETF) accepts electrons from at least thirteen acyl-CoA dehydrogenases. These flavin-dependent dehydrogenases operate in various catabolic pathways such as β-oxidation, amino acid and choline degradation. hETF donates the electrons to a dehydrogenase located in the inner mitochondrial membrane, which in turn feeds the electrons through reduction of ubiquinone into the electron transport chain for ATP production. Recently, the structure of hETF in complex with the human medium-chain acyl-CoA dehydrogenase was reported suggesting that hETF undergoes a major structural change to an open conformation that enables formation of a complex conducive to electron transfer (1). On the other hand, the oxidation reactions of dimethylglycine to sarcosine and further to glycine are catalysed by two very similar FAD-dependent dehydrogenases, dimethylglycine and sarcosine dehydrogenase (DMGDH and SARDH). Detailed biochemical and structural studies have been prevented by the failure to produce the proteins by heterologous expression of the human gene. However, this shortcoming has recently been overcome for DMGDH enabling the first more detailed characterization of the enzyme (2). Since DMGDH adopts a very different structure compared to the enzymes in the family of acyl-CoA dehydrogenases, the current project will focus on the interaction of hDMGDH with hETF. Toward this aim, we have generated two variants of hETF; T266M and R249C, in which the open conformation appears to be favoured enabling crystallisation of the complex with hDMGDH. In addition, we will attempt complex crystallisation with other members of the family of acyl-CoA dehydrogenases, such as isobutyryl-CoA and very long-chain acyl-CoA dehydrogenase to understand how conformational plasticity enables ETF to recognize structurally distinct partners.

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Electron transfer flavoprotein goes moonlighting

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Keywords: Electron Transfer Flavoprotein, Fatty acid oxidation, LYRM5, mitochondrial respiratory complex I.

Mammalian electron transfer flavoproteins (ETF) are heterodimers containing a single FAD, and are located in the mitochondrial matrix. ETF serves as the electron acceptor for nine primary flavoprotein dehydrogenases involved in mitochondrial fatty acid and amino acid catabolism and for two enzymes in choline metabolism. Reduced ETF is re-oxidized by the membrane-bound ETF-ubiquinone oxidoreductase in an electron-transfer pathway linking the primary dehydrogenases with respiratory Complex III. In addition to its role in redox chemistry, ETF now appears to be involved in respiratory Complex I assembly via LYRM5. LYRM5 is a member of the LYRM protein superfamily (LYRMs). LYRMs are basic, approximately 15 kDa polypeptides and contain a conserved tripeptide L-Y-R (leucine/tyrosine/arginine) sequence near the N-terminus. The human genome contains at least ten LYRMs that were predominantly identified as mitochondrial proteins. Human LYRMs are linked with diseases, such as insulin-resistance (LYRM1), muscular hypotonia (LYRM3), deficiency of multiple OXPHOS complexes (LYRM4), to list a few. LYRM5 was shown to be a Complex I phylogenetic profile (COPP) protein by mitochondrial proteomics studies[1].

Recently, mitochondrial protein-interaction-mapping studies identified that LYRM5 strongly interacts with both subunits of ETF[2]. We have performed further biochemical studies using purified proteins of human ETF and LYRM5. The two proteins form a stable complex with a stoichiometry of ETF/LYRM5=1/4. Furthermore, LYRM5 efficiently releases the enzyme-bound FAD upon incubation with ETF, thereby inactivating the enzymatic activity of ETF. Limited trypsin digestion studies and co-expression of LYRM5 with various combinations of ETF domains suggest that LYRM5 binds to ETF at the concave surface of ETF, which is located at the cleft between the two subunits, and overlaps with the FAD binding site. Studies are in progress to determine the exact nature of interactions between ETF and LYRM5, and the non-oxidative role of ETF in Complex I assembly.

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Biphenyl degradation by recombinant cyanobaterium Synechocystis sp. PCC6803 in oligotrophic environments

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Keywords: biphenyl, dioxigenase, cyanobacterium, electron transfer

Bioremediation using microorganisms is an effective way for degradation of polychlorinated biphenyls (PCB) which exists at a low concentration in the environments. Acidovorax sp. strain KKS102 has a biphenyl degradation system composed of BphA1A2, BphA3, BphA4, BphB, BphC, and BphD [1]. In the degradation system, biphenyl is oxidized by a biphenyl dioxygenase (BphA1A2), which requires electrons transferred from an NADH-specific ferredoxin reductase (BphA4) via a Rieske-type ferredoxin (BphA3), and is further degraded to benzoic acid by BphB, BphC, and BphD. Since NADH is supplied mainly from the TCA cycle in bacterial cells, the efficiency of biphenyl degradation is low under oligotrophic environments without a carbon source such as sugars. In order to degrade biphenyl compounds efficiently under oligotrophic environments in cyanobacterial cells using NADPH produced in the plant-type photosynthesis system, the structural gene of an NADPH-specific BphA4 mutant (CRG-mutant) [2] was co-expressed with the structural genes of BphA1A2 and BphA3 under the control of photo-inducible psbE promotor in Synechocystis sp. PCC6803 cells. The biphenyl degradation activity of the mutant cells, which was evaluated from the decrease in the biphenyl concentration in BG-11 culture fluid using HPLC, was higher than that containing the wild-type BphA4 (WT). The production of 2,3-dihydroxybiphenyl was confirmed by using GC-MS as a TMS-derivative. Unexpectedly, the activity of the mutant cells containing only BphA1A2 and BphA3 is also higher than that of the WT, suggesting the existence of cyanobacterial protein(s) which can supply an electron to BphA3 in place of BphA4. Mutant cyanobacterium which coexpressed the structural genes of BphA1A2, BphA3, BphB, BphC, and BphD was also prepared. This mutant showed an obvious increase in benzoic acid and a decrease in the concentration of biphenyl in the culture fluid. These results suggest the usefulness of cyanobacteria for bioremediation of aromatic compounds under oligotrophic environments.

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RosB is the key enzyme of roseoflavin biosynthesis

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Keywords: Roseoflavin, Flavodoxin, AFP synthase, *Streptomyces davawensis*.

The antibiotic roseoflavin (RoF) is the only known natural riboflavin (vitamin B_2) analogue and is active against gram-positive bacteria. RoF is produced by *Streptomyces cinnabarinus* and *Streptomyces davawensis* and can be considered to be an "antivitamin". In RoF biosynthesis the C8 methyl of the precursor riboflavin is replaced by a dimethyl amino group whereby 8-demethyl-8-amino-riboflavin (AF) was postulated to be an intermediate. The first discovered enzyme of roseoflavin biosynthesis was the *S*-adenosyl methionine (SAM) dependent dimethyltransferase RosA which converts AF to RoF. Previous experiments suggested that a single enzyme (RosB) was responsible for the formation of AF. However, when recombinant RosB was tested in an assay mixture containing riboflavin-5'-phosphate (RP) the formation of the predicted final reaction product AF or 8-demethyl-8-aminoriboflavin-5'-phosphate (AFP) was not observed. Instead the compound 8-demethyl-8-formyl-riboflavin-5'-phosphate (HOC-RP) was detected, probably an intermediate of the RosB reaction. How the formyl-group of HOC-RP was replaced by an amino group to give AF or 8-demethyl-8-amino-riboflavin-5'-phosphate (AFP) remained unclear.

The present work was initiated to investigate the predicted oxidation of HOC-RP to 8-demethyl-8-carboxyl-riboflavin-5'-phosphate (HOOC-RP), to identify the amino group donor of the RosB reaction and to shed light on the reaction mechanism of the multi-step enzyme RosB. Heterologous gene expression and biochemical studies revealed that RosB accepts only RP as a substrate and not riboflavin. RosB activity depends on the presence of O_2, thiamine and the amino group donor glutamate. HOOC-RP was found to be an (additional) intermediate of the RosB reaction. The crystal structure of RosB was solved with bound AFP (1.7 Å) and HOC-RP (2.0 Å). RosB is composed of four flavodoxin-like subunits which have been upgraded with specific extensions and a unique C-terminal arm. Structure-based active site analysis was complemented by mutational and isotope-based mass-spectrometric data to propose an enzymatic mechanism.

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Fungal flavoenzymes involved in cellulose degradation

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Keywords: cellobiose dehydrogenase, fungal oxidoreductases, cellulose degradation, lytic polysaccharide monooxygenase

Secreted, FAD-dependent oxidoreductases belonging to the glucose-methanol-choline (GMC) superfamily are ubiquitously distributed in wood decaying fungi. Their exact physiological roles often remain elusive, but have been frequently associated with fungal biomass degradation. Here, we analyzed the structure and function of the extracellular flavocytochrome cellobiose dehydrogenase (CDH, CAZy AA3.1), which is secreted during growth on cellulosic substrates. Using mutational analyses in combination with steady-state and pre-steady state techniques we showed that CDH catalyzes the flavin-dependent oxidation of soluble cellulosic and hemicellulosic sugars, while a flexible cytochrome domain transfers these reduction equivalents from the FAD to external interaction partners[1,2]. Our data support the proposed physiological function of CDH as a reductase for copper-dependent lytic polysaccharide monooxygenase (LPMO, CAZy AA9), which cleaves crystalline cellulose surfaces and boosts the performance of hydrolytic cellulases. A genomic survey showed a high overlap of *cdh* genes with *lpmo* genes in saprotrophic fungi (~70%), but indicated less co-occurrence of these genes in fungi with a limited number of cellulose-hydrolyzing enzymes. Our data also provide evidence that other secreted FAD-dependent enzymes, such as glucose dehydrogenase, could be connected to the degradation of cellulose[3]. These enzymes can activate LPMO indirectly by reduction of low-molecular mass plant phenols, which can serve as efficient redox mediators between LPMO and GMC oxidoreductases. Arguably, fungi secreting GMC oxidoreductases as reduction systems for LPMO can better adapt to changing environmental conditions. The fusion of one of these GMC oxidoreductases with a cytochrome domain evolved CDH as a specific and efficient electron transfer system. These findings may shed light on the abundance and multigenicity of GMC oxidoreductases in fungal genomes and could help understanding their roles in biomass degradation.

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The Bs3 resistance protein – a flavin monooxygenase that triggers cell death

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Keywords: FMO, hydrogen peroxide, plant resistance

Bs3 is a Flavin monooxygenase (FMO) from pepper, which confers resistance to bacterial blight disease, caused by the bacterium *Xanthomonas euvesicatoria* (*Xe*). During infection the bacterium transfers effector proteins into the plant cell that induce Bs3 expression. Bs3 triggers a fast local cell death reaction, the hypersensitive response (HR), which prevents spread of the pathogen.

Within their catalytic cycle, FMOs use NAD(P)H and O_2 to form a stable 4a-hydroperoxy-FAD intermediate. Subsequently one atom of oxygen is transferred to the substrate. In case no substrate is available, FMOs are known to release low amounts of H_2O_2 via a side reaction. [1]

In our experiments, we compare Bs3 to its closest homolog YUCCA6, which is involved in auxin biosynthesis. Despite the high similarity to YUCCA6, Bs3 does not produce auxin. To compare H_2O_2 production of Bs3 and YUCCA6, we purified active protein from *E. coli* and performed H_2O_2 measurements *in vitro*. The results show, that Bs3 consumes more NADPH and produces higher amounts of H_2O_2.

We hypothesize that Bs3 unlike other FMOs does not oxygenate a substrate but exclusively produces H_2O_2 which functions as a signalling molecule and induces a plant immune reaction.

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Isolation and characterization of a thermostableF420:NADPH oxidoreductase from Thermobifida fusca

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Keywords: *Thermobifida fusca*, F_{420}, deazaflavoenzymes, oxidoreductase, nicotinamide adenine dinucleotide phosphate

We report here on the isolation and characterization of a thermostable F_{420}:NADPH oxidoreductase (Tfu-FNO) from *Thermobifida fusca*, being the first F_{420}-dependent enzyme described from this bacterium. Tfu-FNO is a well expressed, highly thermostable enzyme with highest activity at 65 °C. Tfu-FNO, similar to its counterpart in *Streptomyces griseus* [1], most likely acts as an F_{420} reductase at the expense of NADPH inside the cell. The crystal structure of FNO in complex with $NADP^+$ was obtained at 1.8 Å resolution, providing the first bacterial FNO structure. The overall architecture and $NADP^+$ binding site of Tfu-FNO is highly similar to that of the *Archaeoglobus fulgidus* FNO (Af-FNO) [2]. The active site is located in a hydrophobic pocket between an N-terminal dinucleotide-binding domain and a smaller C-terminal domain. Residues interacting with the 2′-phosphate of $NADP^+$ were probed by targeted mutagenesis. Our results show that Thr28, Ser50, Arg51, and Arg55 play an important role in discriminating between $NADP^+$ and NAD^+. Interestingly, the T28A mutant increases the kinetic efficiency more than three-fold as compared to wild type when NADH is used as substrate. The biochemical and structural data presented here provide insights in the molecular recognition of the two cofactors, F_{420} and NAD(P)H by FNO.

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Cytochrome P450 reductase chimera: turn around the hinge

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Keywords: NADPH-cytochrome P450 reductase, salt effect, domains dynamic, hinge domain.

NADPH-cytochrome P450 reductase (CPR) is the redox partner of the cytochrome P450 enzymes, involved in sterol metabolism and xenobiotic detoxification. The CPR contains a membrane anchored FMN domain linked to a FAD domain by a hinge region and a connecting domain (Gutierrez *et al.*, 2003). The CPR oscillates between a compact conformation positioning FMN and FAD in a suitable orientation and distance for electron transfer (locked state) and a series of open conformations (unlocked state) that probably support the reduction of external acceptors. This conformational equilibrium has been shown to be highly dependent on ionic strength, strengthening the hypothesis of the presence of salt interactions at the interface between the FMN and connecting FAD domains (Frances *et al.*, 2015). In the present study, six single mutants and two double mutants of the human CPR targeting the hinge segment have been constructed and their biochemical behaviors analyzed in function of the ionic strength. Reduction of cytochrome c, an unnatural substrate, at various salt concentrations has been quantified in these 8 mutants, introduced either in the soluble or membrane-bound forms of human CPR. All mutants were found active with various efficiencies and a continuum in their optimum salt concentration. The presence of the membrane does not deeply modify the ionic strength profiles, even if some key differences appear in regard of the soluble form. The weight of each position has been implemented in a view of a global open end zip dynamic system between the FAD-FMN domains. This work illustrates that both flexibility and ionic interactions are both controlling the function of the hinge.

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Trifluorosubstrates as mechanistic probes for glycolate oxidase

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Keywords: Carbanion, Glycolate oxidase, Hydride, Trifluorolactate

The family of FMN-dependent *L*-2-hydroxy acid-oxidizing enzymes comprises several well characterized members, such as L-lactate oxidase and monooxygenase, short and long chain hydroxy acid oxidases (GOX and LCHAO), mandelate dehydrogenases, as well as flavocytochrome b_2 (Fcb2), an L-lactate dehydrogenase with a heme domain attached to the flavin domain (FDH). Their crystal structures show a TIM barrel with a highly conserved active site. Thus, family members must use the same chemical mechanism for substrate oxidation. Substrate modelling in the Fcb2 active site suggests that the active-site base, a histidine, could either abstract the hydroxyl proton, thus leading to a hydride transfer, or abstract the C2 proton, with formation of an intermediate carbanion, which would yield electrons to the flavin. It has been shown that the group that picks up the C2 hydrogen has a pK_a of 9.1 in reduced Fcb2 [1]. This value cannot be ascribed to flavin N5-H [2] and as a consequence is incompatible with a hydride transfer mechanism. Nevertheless, a number of authors keep advocating a hydride transfer mechanism. We carried out experiments with fluorinated substrate analogues, namely trifluorolactate (F3Lac) and trifluoropyruvate (F3Pyr). The fluorine atoms should stabilize a carbanion and make hydride transfer difficult. Indeed, F3Lac is an inhibitor and not a substrate for an NAD-linked LDH [3]. We previously compared the reactivity changes between normal and trifluoro substrates for the Fcb2 FDH domain and an NAD-linked LDH [4]. We report here on the reactivity of glycolate oxidase (GOX) with F3Lac and F3Pyr. Altogether, our results cannot be rationalized by a hydride transfer mechanism for the FMN-dependent *L*-2-hydroxy acid-oxidizing enzymes.

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Functional characterization and homology modeling of a novel monofunctional isoform of human FAD synthase

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Keywords: FLAD1, isoform 6, PAPS reductase domain, FAD synthesis.

Alternative splicing of the human FLAD1 gene generates different transcripts coding different isoforms of the enzyme FAD synthase (FADS, EC 2.7.7.2) which catalyses the last step of the biosynthesis of FAD in different sub-cellular compartments [1] (*see also Galluccio-poster at this Meeting*), acting as components of the machinery which delivers the cofactor to the cognate apo-flavoenzymes [2,3].

Silencing of FLAD1 gene in worms impacts on locomotion behavior and causes mitochondrial flavoproteome derangements [4]. Mutations in FLAD1 gene in humans have been recently described as responsible for Riboflavin-Responsive and –Non-responsive Multiple Acyl-CoA Dehydrogenase and combined Respiratory-Chain Deficiency [5].

Isoform 1 and 2 of hFADS have been characterized in some details. They contain an N-terminal molybdopterin binding (MPTb) domain, which has been shown to have Co^{++}-dependent FAD hydrolase activity, and a C-terminal 3'-phosphoadenosine 5'-phosphosulfate (PAPS) reductase domain responsible for the synthesis of cofactor [6].

Other FADS isoforms, their tissue/sub-cellular distributions and structures are at the moment far to be known. We report here some functional features of a recently described novel isoform of FADS, a 320 amino acid long protein, consisting of the sole PAPS reductase domain. The purified protein is a FAD-binding protein able to catalyze FAD synthesis (Vmax about 74 nmol/min•mg), as well as FAD pyrophosphorolysis (Vmax about 9 nmol/min•mg) in a strictly Mg^{++}-dependent manner. We confirm that isoform 6 lacks the ability to hydrolyze FAD and we propose a homology structural model for such isoform, whose functional role and involvement in human physio-pathology will be discussed.

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Drug development for cancer treatment and prevention targeting the human NAD(P)H:quinone oxidoreductase 1

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Keywords: NQO1P187S, cancer, drug, chemotherapy

Human NAD(P)H quinone oxidoreductase 1 is one of the most extensively studied flavoproteins.1,2 Its enzymatic role in the antioxidative defense system as well as the role in stabilising various tumour suppressors and other labile proteins was studied in various laboratories.3,4,5 Due to an increased NQO1 concentration in several tumour cells the use of chemotherapeutics that are activated by NQO1 is already well established.6 In current studies the role of the C609T single nucleotide polymorphism, leading to a proline serine exchange (NQO1 P187S), is investigated in particular. This exchange leads to a reduced activity and stability of the enzyme.7 The influence of the widespread NQO1 P187S polymorphism for a higher risk of cancer development and the failure of different chemotherapies is currently the target of research.8,9

Our former paper7 already provided the structural explanation for the reduced stability and loss of function of NQO1 P187S. Furthermore, we proposed the use of a molecular chaperone to rescue the stability and activity.7 One focus of a possible drug compound, able to support chemotherapy and prevent cancer development, is an increased stability of the target protein. A higher stability will lead to an elevated NQO1 level in the cells and thereby increase the overall enzymatic activity as well as the overall regulatory activity of the tumour suppressors. Another focus of the drug development is the increase of enzymatic activity. Therefore, the use of enzyme inhibitors is not reasonable since the benefit of a higher stability will be undone by a further loss of enzymatic activity. Possible candidates were found with an enhanced *in silico* screening method. A molecular chaperone can already increase the thermal stability of NQO1 as well as the turnover rate significantly. Thus, we have demonstrated that molecular chaperones may be used for the stabilization of unstable proteins.

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Engineering bacterial flavin-monooxygenase for efficient indigo production

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Keywords: indigo, FMO, enzyme engineering, library.

Current industrial indigo dyeing processes involve the chemical synthesis of insoluble indigoid dyes that need to be pre-reduced to become water soluble forms (leuco-indigo) after which it is applied onto a textile. During the dyeing process the cotton yarns are exposed to air to ensure the complete oxidation of the indigoid dye, turning the soluble leuco-indigo into its insoluble form, hence achieving the typical blue color. While indigo blue is currently produced by chemical processes, it would be highly attractive to develop a biotechnological process to produce this popular dye. Recently, a microbial flavin-containing monooxygenase (MeFMO) was discovered that is able to oxidize indole, resulting in the formation of indigo blue1,2.

MeFMO is able to convert indole into indigo blue, but the turnover rate and substrate recognition is rather poor3. This work aims at improving the catalytic performance of the monooxygenase by dedicated enzyme engineering. For achieving a better performing monooxygenase, mutants have been prepared in which residues that form the predicted substrate binding pocket are replaced using site-directed mutagenesis. Mutants have been produced and characterized. Mutations that led to improved catalytic efficiency were combined in order to create a MeFMO mutant that is more effective in indole oxidation. These experiments have resulted in an improved biocatalyst but also provide a better insight into the structural elements that tune the activity of MeFMO and provide hints for further improvement of the monooxygenase as biocatalyst.

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In silico aryl alcohol oxidase engineering – successful stories... and some not so much.

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Keywords: Simulations, Engineering, Oxidoreductases, Aryl alcohol oxidase.

Part of the goals of the multi-partner INDOX European project (www.indoxproject.eu) was to identify, select and tailor oxidoreductases to obtain *ad hoc* industrial biocatalysts with optimized properties. Thanks to the combined effort of experimental and computational researchers we have successfully improved a variety of enzymatic processes, including the industrial synthesis of 1-naphthol[1] and conductive polyaniline[2]. Furthermore, we have developed novel strategies for computer-aided engineering of an aryl alcohol oxidase from *Pleurotus eryngii*. Here, we will present our most recent results and show that often "quick and dirty" computational solutions are more effective than complex calculations.

Due to the accomplishments obtained during the last years[3], we have recently funded an *in silico* protein engineering company – ZYMVOL. Our goals are to democratize the use of computer simulations in protein engineering processes and create consumer awareness to the importance of enzymatic applications in many industrial fields. We employ state of the art, open source software making our in silico solutions truly cost-effective thou capable of predicting the effect of thousands of mutations in few days.

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Crystal structures and atomic model of NADPH oxidase

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Keywords: Membrane protein, Reactive Oxygen Species, Oxidative Stress, Redox Biology.

NADPH oxidases (NOXs) are the only enzymes exclusively dedicated to ROS generation. Dysregulation of these polytopic membrane proteins impacts the redox signaling cascades that control cell proliferation and death. We describe the atomic crystal structures of the catalytic FAD- and heme-binding domains forming the core subunit that is common to the seven members of the family. The domain structures were then docked *in silico* to provide a generic model of NOX family. A linear arrangement of cofactors (NADPH, FAD, and two membrane-imbedded heme moieties) injects electrons from the intracellular side across the membrane to a specific oxygen-binding cavity on the extra-cytoplasmic side. The overall spatial organization of critical interactions is revealed between the intracellular loops on the trans-membrane domain and the NADPH-oxidizing dehydrogenase domain. In particular, the C-terminus functions as a toggle switch, which affects access of the NADPH substrate to the enzyme. The essence of this mechanistic model is that the regulatory cues conformationally gate NADPH-binding, implicitly providing a handle for activating/deactivating the very first step in the redox chain. Such insight provides a framework to the discovery of much needed drugs that selectively target the distinct members of the NOX family and interfere with ROS signaling.

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Rapid enzyme stabilization by computationally designed libraries of HMF oxidase

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Keywords: enzyme stability, in silico design, protein stability engineering, flavoenzyme

HMF oxidase (HMFO) from Methylovorus sp. is a recently characterized flavoprotein oxidase 1. HMFO is able to oxidize 5-(hydroxymethyl)furfural (HMF) into 2,5-furandicarboxylic acid (FDCA). Because HMF can be formed from fructose or other sugars and FDCA is a polymer building block, the oxidase has attracted attention as industrially relevant biocatalyst. The dicarboxylic acid FDCA can be polymerized with ethylene glycol to produce polyethylene furanoate (PEF). This renewable and bio-based polyester can be a valid alternative to the petroleum-based polyethylene terephthalate (PET) thanks to its similar characteristics.

HMFO is a promising biocatalyst for various oxidations and not only for the production of FDCA. The first step to the development of an HMFO with improved catalytic properties is the engineering of the enzyme to enhance its thermostability using the recently developed FRESCO method.

FRESCO (Framework for Rapid Enzyme Stabilization by Computational libraries) is a computational approach to determine thermostabilizing point mutations in a protein structure 2.FRESCO has the potential to become a more valid alternative to random approaches like direct evolution when the protein structure is known. The first step of this computational method is the application of an algorithm based on FoldX and Rosetta ddG calculations to every possible single amino acid mutant to predict the mutant stability. In the following step MD screening is performed in order to eliminate variants with predicted protein flexibility. After this screening the selected mutants are subject to experimental verification for improved TM and preserved catalytic activity. Finally, the combination of stabilizing mutations should lead to highly stabilized variants.

I will present the results obtained by using the FRESCO method: the stability and activity profiles of the generated HMFO mutants.

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The FAD synthetase from *Streptococcus pneumoniae*: an enzyme exhibiting activity-dependent redox requirements

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Keywords: *Streptococcus pneumoniae* FAD synthetase, ATP:riboflavin kinase; ATP:FMN:adenylyltransferase, reduced FMN.

Prokaryotic bifunctional FAD synthetases (FADSs) catalyze the biosynthesis of FMN and FAD, whereas in eukaryotes two enzymes are required for the same purpose. FMN and FAD are key cofactors to maintain the flavoproteome homeostasis in all type of organisms. Here we shed light to the properties of the hitherto unstudied bacterial FADS from the human pathogen *Streptococcus pneumoniae* (*Spn*FADS). As other members of the family, *Spn*FADS catalyzes the three typical activities of prokaryotic FADSs: riboflavin kinase (RFK), ATP:FMN:adenylyltransferase (FMNAT), and FAD pyrophosphorylase (FADpp)[1,2]. However, several *Spn*FADS biophysical properties differ from those of other family members. In particular; i) the RFK activity is not inhibited by the RF substrate, ii) the FMNAT and FADSpp activities require flavin substrates in the reduced state, iii) binding of adenine nucleotide ligands is required for the binding of flavinic substrates/products and iv) the monomer is the preferred state. Collectively, our results add interesting mechanistic differences among the few prokaryotic bifunctional FADSs already characterized, which might reflect the adaptation of the enzyme to relatively different environments. In a health point of view, differences among FADS family members provide us with a framework to design selective compounds targeting these enzymes for the treatment of diverse infectious diseases.

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On the evolution of flavin-dependent enzymes: retracing the steps

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The limited number of protein domains and cofactors that Nature has employed to grow an astounding variety of enzymatic functions, will never cease delighting us. Although enzymes are highly flexible molecules, they form thermodynamically stable structures and therefore their evolutionary trajectories are constrained to a narrow range of pathways. Understanding the evolutionary history of an enzyme family will reveal the complex network of changes that have occurred from ancestral sequences leading to the emergence of extant enzymes. This will allow us to better comprehend enzyme mechanisms and identify the essential subset of residues responsible of functional differences between family members. Moreover, dynamics of enzyme evolution can unveil the functional constraints and contingencies on how a new enzyme function arises, and ultimately what makes an enzyme to display a certain activity.

In this context, the evolutionary history of two different enzyme families (meaning, a set of enzymes displaying the same fold and active site features and thus likely to share a common ancestor) was studied. Flavin-dependent monooxygenases are a functionally diverse family, including aromatic hydroxylases, Baeyer-Villiger monooxygenases and epoxidases. By integrating data from sequences, 3D structures, multidomain architectures, chemistry and phylogenetic inferences, we were able to unveil a complex set of factors influencing the evolution of this family and leading to its current diversity[1,2]. By a different approach, we have focused on resolving the evolutionary relationships of enzymes using F_{420} and/or FMN as cofactor. Particularly, how reductases and dehydrogenases, with a focus on FGDs (glucose-6-phosphate dehydrogenases)[3], emerged from an FMN-dependent ancestor and diverged into the current functional variety, has been addressed. Moreover, by using the ancestral sequence reconstruction approach and resurrecting enzymes in the lab, the emergence of the dehydrogenase function has been traced.

These examples show how phylogeny and bioinformatics bridge the gap between biochemistry and molecular evolution and provide insight on how a new enzyme-function emerges. This offer valuable information for enzymology related research and may facilitate more effective enzyme discovering and enzyme engineering efforts.

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Kinetics and thermodynamics in ligands binding as FMN production determinants in bifunctional FAD synthetases

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Keywords: Riboflavin kinase, FAD synthetase, stopped-flow, ITC.

Prokaryotic bifunctional FAD synthetases (FADSs) exhibit ATP:riboflavin kinase (RFK) activity in its C-terminal module and FMN:ATP:adenylyltransferase (FMNAT) activity in the N-terminal one[1]. The RFK activity of the *Corynebacterium ammoniagenes* enzyme (*Ca*FADS) is highly regulated by RF substrate inhibition as well as by reaction products inhibition[2,3]. Contrary to *Ca*FADS, the homologous enzyme from the human pathogen *Streptococcus pneumoniae* (*Spn*FADS) does not show inhibition of its RFK activity and its FMNAT activity only works with the reduced FMN. Such dissimilarities suggest different mechanisms contributing to regulate the flavin and flavoproteome cellular homeostasis. Considering also the FADSs potential as exploitable targets in the design of selective inhibitors to treat infectious diseases, the comparative study of the RFK activity mechanisms in these two enzymes is a topic worthy of study.

Here we shed light on the regulation of the RFK activity of *Ca*FADS through the study of the role played by its substrates and products as inhibitors. In addition, the pre-steady-state kinetic and thermodynamic evaluation of binding of substrates and products to *Ca*FADS and to *Spn*FADS, collectively with the available structural information, allows depicting key differential aspects in the induced fit of substrates to reach the RFK catalytic complex. Our data reveal as the main cause for the inhibition in *Ca*FADS, the formation of all possible non-competent complexes with substrates and products, which compete, both kinetically and thermodynamically, with the catalytically competent one. Thus, a complex network of non-competent interactions provides with an intricate regulatory mechanism that allows the suitable production of flavins according to the *C. ammoniagenes* necessities. Such strategy agrees with selective inhibition of key enzymes being a common tool to regulate metabolic pathways. On the other hand, binding of the RFK reaction substrates to *Spn*FADS is by far the faster and more favorable process. Furthermore, in *Ca*FADS the Mg^{2+}-dependent-concerted allocation of substrates is required for catalysis, while a sequential binding order, also cation-dependent, is found in *Spn*FADS.

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LAAD: a novel promising industrial biocatalyst

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Keywords: L-amino acid deaminase, flavoenzyme, biocatalyst, enantiomeric resolution.

L-amino acid oxidases (LAAOs) are flavoenzymes that could be used as biocatalysts in "green processes" for the production of enantiomerically pure D-amino acids and α-keto acids. In particular, D-amino acids are high value-added chemicals for the fine chemistry and pharmaceutical industry since they are used for the synthesis of pharmaceutical drugs, antibiotics and insecticides. Enantioselective biocatalysis or dynamic kinetic resolution of racemic mixtures is a competitive approach both in terms of yields and costs in comparison to classical asymmetric synthesis. In recent years several protocols for the production of optically pure L-amino acids using an enantioselective D-amino acid oxidase have been set-up [1, 2]. Unfortunately, these processes cannot be adapted for the production of the opposite enantiomer because of the drawbacks in recombinant production of a LAAO activity [3].

An alternative to LAAO is represented by L-amino acid deaminase from *Proteus myxofaciens* (PmaLAAD). This membrane flavoenzyme catalyzes the oxidative deamination of L-amino acids to the corresponding α-keto acids without hydrogen peroxide production. PmaLAAD has a broad substrate specificity, being active on aromatic or aliphatic L-amino acids and on several unnatural amino acids of biotechnological relevance such as L-DOPA and substituted alanines. The knowledge of the 3D structure of PmaLAAD renders this enzyme the ideal scaffold for the evolution of a suitable biocatalyst for industrial applications [4].

We exploited the N-terminal His-tagged full-length variant of PmaLAAD to catalyze the deracemisation (e.g., on D,L-Met or D,L-naphtylalanine) or the dynamic kinetic resolution (e.g., on L-4-nitro-phenylalanine) of different natural and unnatural amino acids.

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Characterization of a bacterial pyranose 2-oxidase from *Arthrobacter siccitolerans*

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Keywords: Flavoprotein, Glucose-methanol-choline family of oxidoreductases, Hydrogen peroxide forming enzymes, Carbohydrate chemistry

In this study we provide the first biochemical characterization of a bacterial pyranose 2-oxidase (AsP2Ox) from *Arthrobacter siccitolerans*. The enzyme catalyzes the oxidation of several aldopyranoses at the C-2 position, coupling it to the reduction of dioxygen to hydrogen peroxide. Pyranose 2-oxidases belong to the glucose-methanol-choline oxidoreductase family. A structural model based on the known X-ray structure of P2Ox from *Phanerochaete chrysosporium* supports that AsP2Ox shares structural features with well-characterized fungal P2Oxs. The gene coding for AsP2Ox was cloned and heterologously expressed in *Escherichia coli*. The purified recombinant enzyme is a 64-kDa monomer containing a non-covalently bound flavin adenine dinucleotide (FAD) cofactor, distinct features as compared with fungal counterparts that are ~ 270 kDa homotetramers with covalent-linked FAD. AsP2Ox exhibits a redox potential of −50 mV, an optimum temperature of 37°C and an optimum pH at 6.5. AsP2Ox oxidizes D-glucose at the highest efficiency, using additionally D-galactose, D-xylose, L-arabinose and D-ribose as electron donors, coupling their oxidation to the reduction of both dioxygen and 1,4-benzoquinone. AsP2Ox shows a relatively low thermal stability with a melting temperature (T_m) of 43°C and a half-life ($t_{1/2}$) at 40°C of 25 min.

Currently, studies are in progress to improve the kinetic properties of this enzyme by directed evolution and one variant was already found with higher activity for the oxidation of D-glucose than the wild-type enzyme. With this work we are expanding the repertoire of bacterial oxidoreductases with importance in biotechnological and diagnostic applications.

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Monolignol oxidoreductases from the berberine bridge enzyme-like protein family

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Keywords: monolignol dehydrogenases, Arabidopsis thaliana, berberine bridge enzyme like enzyme, BBE

Plant genomes contain a large number of genes encoding berberine bridge enzyme (BBE)-like enzymes. The number of these genes in individual plant species varies considerably from two genes in the moss *Physcomitrella patens* to 65 in the western poplar (*Populus trichocarpa*). Despite the widespread occurrence and abundance of this protein family in the plant kingdom, their biochemical function remains largely unexplored.

In the case of *Arabidopsis thaliana*, 28 BBE-like genes were identified. These 28 genes form 7 phylogenetic groups with a distinct active site composition implying different catalytic activity and substrate specificity. Recently, two members of the BBE-like proteins in *Arabidopsis thaliana* (*At*BBE) were identified as monolignol oxidoreductases implicating these enzymes in the manipulation of the extracellular monolignol pool prior to lignification[1].

The aim of our work is to express and characterize additional putative *At*BBE monolignol oxidoreductases. Toward that goal, we combine bioinformatics, protein expression, protein characterization and protein crystallography.

Additionally, we aim to elucidate the physiological role of the monolignol oxidoreductases in *planta*. Therefore, our biochemical approach is complemented by the characterization of the respective monolignol oxidoreductase knock out plants. We are particularly interested in changes of the lignin structure and content. The expression patterns of different monolignol oxidoreductases will be visualized employing the GUS-reporter system.

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Ultrafast kinetic studies reveal effects of electron transfer and charge recombination on the lifetime of flavin semiquinone

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Keywords: Transient Absorption Spectroscopy, Electron Bifurcation, Flavin, Electron Transfer.

Flavin-based electron transfer bifurcation is emerging as a fundamental and pervasive mechanism for conservation and deployment of electrochemical energy in enzymatic systems. It is believed that thermodynamic destabilization of a flavin semiquinone species is essential, so it is expected that this species should be short-lived. We now demonstrate that a short-lived anionic flavin semiquinone (ASQ) is not sufficient to infer bifurcating activity, although it may be necessary. To address this question, we have used transient absorption spectroscopy to compare the rates and mechanisms of decay of ASQ generated photochemically in bifurcating and non-bifurcating flavoproteins. We find that different mechanisms dominate in mediating decay of ASQ, producing lifetimes ranging over two orders of magnitude. Capacity for electron transfer among redox cofactors vs. charge recombination with nearby donors succeeds in explaining the range of ASQ lifetimes we observe. Our data support a model wherein efficient electron propagation can be an explanation for the short lifetime of the ASQ of bifurcating NADH-dependent ferredoxin-NADP+ oxidoreductase I, and a diagnostic ingredient of capacity for electron bifurcation.

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Inhibitor-induced *in situ*-chaperone therapy: a novel strategy for treating MCAD and VLCAD deficiencies

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Keywords: MCAD, VLCAD, trimetazidine, phenylbutyrate

Background: The increase in thermal stability of enzymes and their protection against proteolysis induced by ligand binding are well documented structural phenomena. Thermal stability of medium-chain acyl-CoA dehydrogenase (MCAD) is enhanced by substrate binding1. Providing alternative MCAD substrates may produce a chaperone effect provided amounts administered are controlled to avoid toxic metabolites. Phenylbutyryl-CoA has previously been reported as an MCAD substrate2, and treatment with phenylbutyrate was predicted to improve stability of mutant MCAD protein in cells with MCAD deficiency. In addition, it is hypothesized that the presence of modulating intermediates that target MCAD could have a similar effect acting as *in situ*-chaperones. Trimetazidine (TMZ) is an inhibitor of long-chain 3-ketoacyl-CoA thiolase, that catalyzes the last step of long-chain fatty acids' β-oxidation. This is hypothesized to cause accumulation of β-oxidation intermediates including long- and medium-chain *trans*-Δ2-enoyl-CoAs, the products of very long-chain acyl-CoA dehydrogenase (VLCAD) and MCAD.

Method: Fibroblasts from patients homozygous for the MCAD K304E variant were treated with phenylbutyrate and TMZ and tested for *in situ* enzyme presence and activity. Fibroblasts from patients with various VLCAD missense mutations were treated with TMZ and tested for enzyme presence and activity, and for acylcarnitines profile in the media.

Results: Dose response, in terms of proportional increase of enzyme presence and activity, was detected in fibroblasts deficient in MCAD treated with phenylbutyrate or TMZ, and in cells deficient in VLCAD treated with TMZ. The acylcarnitine profile of cells with VLCAD deficiency indicate an initial decrease before increasing at higher drug concentrations.

Conclusion: MCAD and VLCAD variants are amenable to chaperone therapy. While TMZ causes an increase in variants presence and activity of either protein, the increase varies depending on the degree of instability of the mutants, and so therapy with TMZ should be considered in terms of a combination with other therapy strategies.

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Expression and structural determination of an alcohol oxidase from *Phanerochaete chrysosporium*

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Keywords: flavin-dependent oxidases, glucose-methanol-choline oxidases, steady-state kinetics, X-ray structure.

The recently described alcohol oxidase from the white-rot basidiomycete *Phanerochaete chrysosporium* (PcAOX) was heterologously expressed in *Escherichia coli* as a fusion protein. The recombinant enzyme was obtained with high yield and displayed high thermostability in commonly used buffers. Steady-state kinetics revealed that the enzyme is highly active towards methanol and ethanol (k_{cat} = 18 and 19 s−1, respectively), but showed very limited activity towards glycerol. The crystal structure of the native homo-octameric PcAOX was determined at 2.40 Å resolution. The subunit arrangement and the secondary architecture of each monomer is highly conserved in PcAOX as compared to its homolog AOX1 from *Pichia pastoris* (PpAOX1). Similar to PpAOX1, the catalytic center is a remarkably solvent-inaccessible cavity located at the *si* site of the flavin cofactor. The active site is intriguingly small in size, which could well explain the observed preference for methanol and ethanol as best substrates. With the available expression system and structural information, PcAOX represents a potential candidate for protein engineering towards more industrially attractive substrates, for instance, glycerol—a major byproduct of biodiesel production.

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Reactions of lumiflavin-N5-oxide

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Keywords: flavin-N5-oxide, lumiflavin, oxygen

Flavin-N5-oxides have long been known as the penultimate synthetic intermediates in one laboratory route to flavins. Three decades ago, flavin-N5-oxides were also considered as possible enzymatic intermediates in flavin-oxygen reactions, but were rejected due to decisive evidence demonstrating flavin hydroperoxides. Recently, two enzymes have been reported to involve the N5-oxide1,2,3, and no doubt many more will be discovered soon. Little is known about the chemistry available to N5-oxides – how they are formed, or what they react with. Understanding their basic bioorganic chemistry is essential for defining their versatility in enzymology; initial efforts are reported here.

Lumiflavin-N5-oxide was deoxygenated in aqueous buffer containing 2 M formamide as co-solvent by a variety of model N- and S-nucleophiles, as well as NADH. Interestingly, in the reaction of a model tertiary amine – MOPS buffer – strong evidence was obtained for an intermediate on the path from lumiflavin-N5-oxide to lumiflavin. Aerobic solutions of the N5-oxide were apparently stable, as judged from the absorbance spectrum, but removal of O_2 caused the conversion of lumiflavin-N5-oxide to lumiflavin. Thus, O_2 oxidizes the initial reaction product of MOPS and lumiflavin-N5-oxide, regenerating lumiflavin-N5-oxide. The high-potential dye DCIP could also intercept the putative intermediate and prevent the depletion of the N5-oxide / formation of lumiflavin. Other aliphatic amines – primary, secondary, and tertiary – converted lumiflavin-N5-oxide to lumiflavin. Interestingly, Tris buffer reacted; a tertiary carbon is attached to the nitrogen of this primary amine. Because oxidation to an imine is not possible with Tris, the oxidation product is likely to be the hydroxylamine, suggesting that reactions with amines involve only the nitrogen.

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Novel Baeyer-Villiger monooxygenases discovered by genome mining in a *Streptomyces* from the Atacama Desert

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Keywords: Baeyer-Villiger monooxygenases, genome mining, extremophiles, Type II FMOs

Baeyer-Villiger monooxygenases (BVMOs) are a prominent example of oxidative enzymes, which catalyze not only Baeyer-Villiger reactions, but also halogenations, epoxidations and sulfoxidations with high chemo-, region- and stereoselectivity. Among BVMOs, the NADPH-dependent Type I BVMOs have been the most studied. However, a novel group of BVMOs that display relaxed coenzyme specificity, by accepting both nicotinamide cofactors, was recently described and named type II FMOs. This novel subclass of BVMOs are of great potential for biocatalytic industrial applications, since the non-phosphorylated alternative, NADH, is ten times less expensive, more stable and more predominant within cells than NADPH. In order to study novel type II FMOs, we search in the proteome of *Streptomyces leeuwenhoekii*, an Actinobacteria isolated from the driest desert in the world: the Atacama Desert in the north of Chile. Genome mining using previously described typifying motifs[1, 2] revealed two putative Type II FMOs and also three putative Type I BVMOs. A phylogenetic tree was generated including representative enzymes from both subclases and the five BVMOs from *S. leeuwenhoekii*. As expected, the two putative Type II FMOs clustered with this novel group of enzymes. The genomic DNA from *S. leeuwenhoekii* was used as template to amplify the 5 putative BVMO sequences. Genes were cloned using a ligation free method and heterogously expressed as soluble proteins using the pCRE2/*E. coli* expression system. Cofactor and substrate specificities were studied for each of the proteins by using NAD(P)H depletion assay, confirming the dual cofactor property of the novel Type II FMOs and Baeyer-Villiger activity of the five enzymes. Next steps include the study of the novel enzymes in terms substrate enantioselectivity, kinetics parameters, and protein structure.

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Using the flavin monooxygenase StyA to produce aromatic chiral azido alcohols

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Keywords: styrene monooxygenases, nicotinamide coenzyme biomimetics, chiral epoxides, two-component flavin monooxygenases.

The flavoprotein styrene monooxygenase StyA can catalyse epoxidation and sulfoxidation reactions using molecular oxygen with excellent chemo- and enantioselectivity. StyA uses the pyridine nucleotide coenzyme NADH as an electron donor to reduce the flavin (FAD) through a reductase (StyB). A synthetic nicotinamide coenzyme, 1-benzyl-1,4-dihydronicotinamide (BNAH), can replace the native NADH/reductase system to reduce the FAD in solution. This StyA/BNAH system gave the same enzyme activity as with the natural StyA/NADH/reductase system, thus demonstrating a highly simplified electron transport to the enzyme.[1]

A chemo- and bi-enzymatic cascade was developed using StyA/BNAH starting from aromatic vinyl derivatives for the preparation of chiral aromatic azido alcohols. As an example, starting from styrene and employing azide as nucleophile, the enantiomerically pure epoxide intermediate was transformed into the desired chiral product. A variety of substituents can be present on the aromatic ring (Br, Cl, F, Me, MeO) and on the α- or β-carbon (Me, giving two chiral centers) of the substrates without affecting the reaction conditions.

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A decarboxylase depending on prenylated flavin for the regioselective *para*-carboxylation of phenols

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Keywords: prenylated flavin, *para*-(de)carboxylation, phenols, mechanism

In recent years, various concepts of enzyme-catalyzed carboxylation processes, in particular for electron-rich (hetero)aromatic compounds have been developed as attractive 'green' alternative to chemical methods.1 Bio-carboxylation strategies for the regioselective *ortho*- and β-carboxylation of phenols and hydroxystyrenes, respectively, have been successfully established by running decarboxylases in the reverse carboxylation direction at the expense of bicarbonate as CO_2 source.2 Our research efforts to expand the biocatalytic toolbox towards the elusive regio-complementary para-carboxylation of phenols peaked in the discovery of 3,4-dihydroxybenzoic acid decarboxylase from *Enterobacter cloacae* (3,4-DHBD)3 depending on the recently discovered prenylated flavin (prFMN)4 as cofactor. Results from quantum-mechanical calculations, mutational- and substrate studies led us to propose a mechanism involving a transient, monocovalent phenol-cofactor adduct and thus differs from the mechanism for prFMN-dependent (de)carboxylation of cinnamic acids.5

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Structural and mechanistic insights into prFMN dependent aromatic reversible (De)carboxylases

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Keywords: prFMN, Modified Flavin, Decarboxylase, Protein Structure,

The UbiD/Fdc1 family are a wide spread and diverse family of enzymes capable of catalyzing the reversible (de)carboxylation of aromatic or unsaturated aliphatic substrates. These enzymes were recently discovered to be dependent on a novel prenylated form of FMN (termed prFMN) where a prenyl group is attached to the N5 and C6 positions of the isoalloxazine ring system such as to create a 4th non-aromatic ring[1]. In the case of the fungal Fdc1 enzymes prFMN has been shown to support (de)carboxylation via 1,3-dipolar cycloaddition between the unsaturated aliphatic substrate (a dipolarophile) and the azomethine ylide (a well known dipole) of the modified cofactor[2]. Whilst this mechanism has been generally accepted for prFMN dependent enzymes that act on aliphatic substrates, the involvement of 1,3-dipolar cycloaddition in the (de)carboxylation of aromatic substrates remains controversial as it would require transient dearomatisation of the ring system. Here we report the application of a number of structural, biophysical and biochemical techniques to further elucidate the mechanism of these enzymes.

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Investigation on mechanism and substrate specificity of flavin-dependent tryptophan 6-halogenase

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Keywords: tryptophan 6-halogenase, flavin-dependent tryptophan halogenase, halogenation, halogenated compounds

Tryptophan 6-hlogenase (Thal) is an FADH--utilization enzyme. This enzyme catalyses halogenation of tryptophan (Trp) at the C6-position. The reaction requires FADH-, oxygen, chloride ion and tryptophan as substrates. The reaction of an aerobic solution of Thal and FADH- resulted in formation of FAD(C4a)-OOH intermediate that can be detected by stopped-flow spectrophotometry. However, the reaction of pre-mixed Thal and FADH- did not show formation of FAD(C4a)-OOH intermediate. In the presence of halide ions except fluoride, FAD(C4a)-OOH decayed to yield FAD(C4a)-OH, indicating that halides may interact with FAD(C4a)-OOH to produce FAD(C4a)-OH. Specificity of Thal on aromatic substrates and halides was investigated using multiple turnover reactions. Products were analyzed by HPLC-DAD/MS. Besides tryptophan, Thal can also use other aromatic compounds as substrates. Different aromatic substrates have different specificity toward different halides.

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Construction of eukaryotic bioreporter systems based on bacterial luciferase

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Keywords: Bacterial luciferase, Bioluminescence, Bioreporter gene, BNAH

Bacterial luciferase (Lux) catalyzes oxidation of reduced flavin and long chain aldehyde by molecular oxygen to yield oxidized Flavin, carboxylic acid and water as products. This reaction concomitantly generates blue-green light. As substrates of Lux are cheaper than many substrates of other bioluminescent reactions, Lux is potentially useful as a bioreporter system for molecular biology research. To develop Lux as a reliable bioreporter to be used in eukaryotic systems, the fusion gene of Lux_Vc encoding for a monocistronic single peptide and mammalian expression system were constructed. The expression of Lux in embryonic kidney (HEK293T) and human hepatocyte (HepG2) cell lines were optimized. The results showed that the fusion Lux_Vc was successfully expressed in HEK293T and HepG2 cells. Interestingly, the fusion Lux_Vc system has very low bioluminescent signal background compared to those from firefly luciferase under the same expression condition. A higher signal to noise ratio of Lux_Vc makes it suitable to be used in bioreporter applications. To explore other possibilities of providing reduced flavin for Lux, 1-benzyl-1,4-dihydronicotinamide (BNAH) was used to generate reduced flavin to be used as a substrate for Lux_Vc. The preliminary results have shown that the system works well as an reduced FMN generating system.

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Mechanistic studies of flavin-dependent monooxygenase and reductase for detoxification of toxic aromatic compounds

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Keywords: flavin-dependent monooxygenase, dehalogenase, quinone reductase, transient kinetics, halogenated phenols (HPs), nitrophenols (NPs).

Halogenated phenols (HPs) and nitrophenols (NPs) are widely used as agrochemicals. These compounds are toxic and their accumulation is a major environmental concern. In this work, reaction mechanisms of the enzymes HadA and HadB from biodegradation pathway of *Ralstonia pickettii* DTP0602 were studied. HadA, a flavin-dependent monooxygenase, is a key enzyme for initial steps of biodegradation. We investigated and showed that the purified HadA can catalyze two consecutive steps of oxidative dehalogenation (hydroxylation and group elimination) of chlorophenols first at position 4 and then at position 2 to generate hydroquinone and hydroxyquinol products, respectively. HadA has broad substrate utilization because it can use various HPs and NPs as substrates. Mechanism of HadA with 4-chlorophenol (4-CP) as substrate was investigated using transient kinetics techniques1. C4a-hydroperoxy-FAD and C4a-hydroxy-FAD were found to be intermediates in this reaction. Results show that C4a-hydroperoxy-FAD forms first before substrate binding. Product formation is the rate-limiting step of the overall reaction with hydroxylation step occurs prior to group elimination. Quantitative-structure activity relationship of HadA reaction show that rate of hydroxylation is dependent on deprotonation of phenol to form a phenolate substrate while the rate constant of group elimination is dependent on strength of C-X bond and the stability of leaving group. HadB is an FMN-bound enzyme. Investigation of the purified HadB using steady-state and pre-steady state kinetics shows that HadB is a quinone reductase, not a flavin reductase. The reaction of HadB with NADH and menadione (as quinone substrates) occurs via a ping-pong mechanism. This is the first investigation of the kinetic mechanism of a two-component flavin-dependent monooxygenase (HadA) and quinone reductase (HadB) that can catalyze oxidative group elimination of various HPs and NPs. This will serve as the basis for future investigation of enzyme variants that will be useful for applications in bioremediation.

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Photoregeneration of NAD⁺ using LED light sources

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Keywords: cofactors regeneration, blue LEDs, dehydrogenase, flavins

Oxidation reactions employing alcohol dehydrogenases are experiencing an increased interest in the field of biocatalysis.[1,2] With an in situ regeneration system of NAD⁺, these nicotinamide-dependent oxidoreductase enzymes are more suitable. The photocatalytic oxidation of NADH using a flavin photocatalyst and a simple blue LED light source is reported.[3] This in situ NAD⁺ regeneration system can be used to promote biocatalytic, enantioselective oxidation reactions. Compared to the traditional use of white light bulbs this method enables very significant reductions in energy consumption and CO_2 emission.[4]

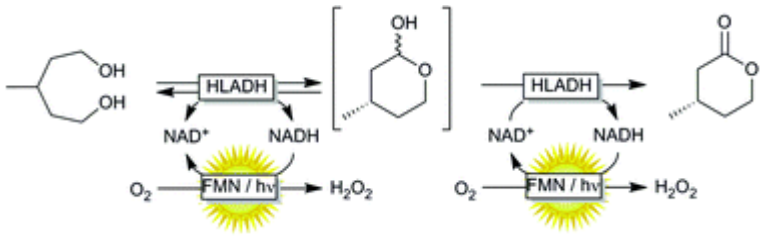

Figure 1. Oxidative lactonization of meso-3-methyl-1,5-pentanediol to (S)-4-methyltetrahydro-2H-pyran-2-one using horse liver alcohol dehydrogenase (HLADH) and photocatalytic, aerobic regeneration of NAD+.

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Formate oxidase (FOX) activity is governed by autocatalytic formation of 8-formyl FAD exhibiting unusual properties

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Keywords: 8-formyl FAD, formate oxidase, glucose methanol choline, novel cofactor.

Formate oxidase (FOX; E.C. 1.2.3.1) from *Aspergillus oryzae* has been identified as the first and only member of the glucose-methanol-choline (GMC) oxidoreductase superfamily of enzymes to oxidize a carbonic acid. Additionally, wild-type FOX has been shown to exhibit an unusual UV absorption spectrum that was due to a non-covalently bound 8-formyl FAD in place of the typical FAD cofactor present in most GMC oxidoreductases. Although the presence of an enzyme bound 8-formyl FMN has been reported previously as a result of site-directed mutational studies on lactate oxidase (LOX), FOX is the first reported case of 8-formyl FAD being present in a wild-type enzyme. Since the formation of 8-formyl FMN in LOX has been shown previously to result in complete inactivation of the enzyme, the presence of 8- formyl FAD in FOX was proposed to be an artifact. Therefore, both the formation and role of the 8-formyl FAD cofactor in formate oxidase was investigated through the use of steady-state kinetics, rapid-reaction kinetics, kinetic isotope effects, site-directed mutagenesis, ICP analysis, UV and fluorescence spectrometry, LCMS, electron paramagnetic resonance (EPR) spectroscopy, analytical ultracentrifugation (AUC), and light-exposure studies. Surprisingly, the results from these studies not only indicate that 8-formyl- FAD is present in the active form of FOX but that its autocatalytic formation is crucial for activity. As a result, formate oxidase serves as the first enzyme reported to have an active 8-formyl FAD as a cofactor. The FOX bound 8-formyl FAD was also shown to form a highly stable anionic semiquinone when exposed to light.

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The biological role of iodotyrosine deiodinase in an organism that is not known to require iodide or iodotyrosine

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Keywords: dehalogenase, *Drosophila*, fertility, spermatogenesis

The flavoprotein iodotyrosine deiodinase (IYD) was first discovered in mammals and named for its ability to salvage iodide from mono- and diiodotyrosine that form during biosynthesis of thyroid hormones (triiodothyronine and its prohormone, tetraiodothyronine or thyroxine). IYD is vital to human health and patients with defective IYD suffer from thyroid disease. Surprisingly, this enzyme is also found in genomes of all metazoans and a limited number of bacteria regardless of their known requirements for iodide. Deiodinase activity was anticipated from the conservation of key residues within the active site1 and confirmed by expression of homologs from 13 representative organisms. Since IYD is also capable of debrominating and dechlorinating their respective halotyrosines,2,3 the enzyme may act as a general dehalogenase in many organisms. Yet, the biological function and processing of halotyrosines in general are not well characterized in nature.

Drosophila melanogaster was selected as a model organism to identify the role of IYD in insects. No data is available to suggest that iodide is required for *Drosophila* and only recently has its requirement of bromide been confirmed.4 IYD from *Drosophila* has now been expressed heterologously and shown to mimic the behavior of human IYD in regards to dehalogenation of various halotyrosines.3 Flybase suggests high expression of IYD in testes and this has been confirmed by in situ hybridization. Deletion and mutation of this enzyme using CRISPR/Cas9 dramatically suppressed fertility over time after an initial mating. This effect was most prominent for mutation and deletion in females despite the greater expression of IYD in males. The biological basis of these observations is now being examined by confocal microscopy of both *Drosophila* testes and ovaries.

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Crystal structure of a xylooligosaccharide oxidase (Xylo) from *Myceliophthora thermophila* C1

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By inspection of the predicted proteome of the fungus *Myceliophthora thermophila* C1 for vanillyl-alcohol oxidase (VAO)-type flavoprotein oxidases, a putative oligosaccharide oxidase was identified. The protein was found to contain a bicovalently bound FAD cofactor. Several mono- and oligosaccharides could be identified as substrate. The enzyme exhibits a strong substrate preference towards xylooligosaccharides, hence it is named xylooligosaccharide oxidase (XylO). Oxidation of xylobiose occurs at C1, yielding xylobionate as product.

By elucidation of several XylO crystal structures (in complex with a substrate mimic, xylose and xylobiose), the residues that tune the unique substrate specificity and regioselectivity could be identified. XylO belongs to CAZy (Carbohydrate Active enZymes) family AA7 (Auxiliary Activity 7). The discovery of this novel oligosaccharide oxidase reveals that the VAO-type flavoprotein family harbors oxidases tuned for specific oligosaccharides.

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Kinetics of the light-driven reduction of 5-deaza riboflavins

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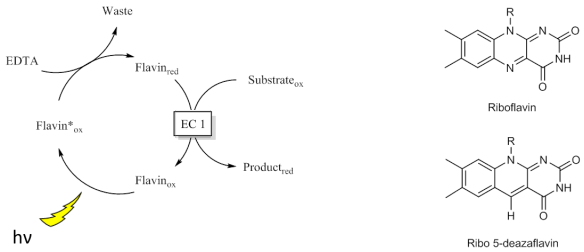
Keywords: Deazaflavins, Biocatalysis, Photochemistry, Cofactor regeneration.

When excited by visible light, flavins are able to accept electrons from sacrificial electron donors like EDTA, ascorbic acid or amino acids.[1] Subsequently, the reduced flavins can reduce an array of oxidoreductases. This makes them interesting photo-active mediators for enzymatic redox reaction (scheme 1, left). Using these compounds to this end could replace the need of complicated regeneration systems for expensive cofactors like nicotinamide cofactors, thereby making this class of enzymes more available for industry. However, one adverse characteristic of this system is the uncoupling of the electron supply from the enzyme reaction. The reduced flavins are able to react with molecular oxygen thereby forming hydrogen peroxide, which is a waste of the electrons.[2] Also, hydrogen peroxide can destabilize the enzymes. To prevent this uncoupling, deazaflavins (scheme 1, right) could be used as the mediator instead of the flavins. It is known that deazaflavins are also able to function as light-activated mediators using sacrificial electron donors.[3] Moreover, the reduced deazaflavins are stable in the presence of oxygen, which could make them an excellent replacement for the natural flavins. However, little is known about the electron transfer rates under different conditions when these compounds are used. Therefore, the behaviour of the deazaflavins is investigated in the current study, in order to understand and to use these compounds for enzymatic redox reactions.



Scheme 1: left: the proposed regeneration system for flavins in order to participate in enzyme catalysed reactions. Right: The molecular structures of riboflavin and 5-deazariboflavin.. R, in this case, is a ribityl-group

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Radicals and radical pairs in flavoproteins

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Keywords: flavin radicals, flavoproteins, cryptochromes, EPR spectroscopy

Flavin semiquinones are common intermediate redox states in flavoproteins and hence, detailed knowledge of their chemical nature is required for a full understanding of their versatility in catalysis [1]. In this contribution, we use a number of (time-resolved) molecular spectroscopy methods to explore flavin radicals in various protein and non-protein environments.

Three examples will be presented: First, we were able to stabilize anionic and neutral flavin radicals in aqueous solutions and characterize both states using electron paramagnetic resonance (EPR) methods. Second, the radical state of a recently discovered flavin intermediate in monooxygenases, the flavin-N(5)-oxide, was analyzed in detail. The resulting electron-spin density distribution lead to a classification in between a “normal” flavin cofactor radical and a well-established nitroxide spin label. Finally, short-lived paramagnetic intermediates of flavin-dependent cryptochrome photo- and magnetoreceptors were characterized by time-resolved EPR methods of all flavors [2]. Here, details in electron-transfer pathways could be revealed [3], which have direct impact on the ability of insect cryptochromes to perceive magnetic fields [4].

The work presented has been performed in collaboration with:

A. Bacher, B. Illarionov, M. Fischer (Hamburg University); P. J. Hore, C. Timmel and S. Mackenzie (Oxford University); .R. Batcha and R. Teufel (Freiburg University)

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Reversibility of the reactions catalyzed by ferredoxin-NAD(P)$^+$ oxidoreductases from phototrophic and heterotrophic bacteria

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Keywords: charge transfer, hydride transfer, stopped-flow, photosynthesis

Ferredoxin-NAD(P)$^+$ oxidoreductase (FNR) is a soluble flavoprotein catalyzing the reversible redox reactions between NAD(P)$^+$/NADPH and small iron sulfur protein ferredoxin. FNRs from phototrophic green sulfur bacterium *Chlorobaculum tepidum* (*Ct*FNR), purple non-sulfur bacterium *Rhodopseudomonas palstris* (*Rp*FNR) and heterotrophic gram-positive bacterium *Bacillus subtilis* (*Bs*FNR) are homo-dimeric proteins with significant structural homology to the bacterial NADPH-thioredoxin reductases and structurally distinct from the other monomeric FNRs of cyanobacteria and prokaryotes. In this presentation, formations of charge transfer (CT) complex and kinetics of hydride transfer process in NADP$^+$/NADPH reduction/oxidation reactions are studied by pre-steady-state kinetic analyses using stopped-flow spectrophotometry.

Mixing oxidized FNRs with NADPH yielded a formation of CT complexes within the dead time then the reduction of FNRs followed. *Bs*FNR was almost fully reduced at equilibrium whereas *Rp*FNR and *Ct*FNR were reduced in part even in the presence of ~50-fold excess NADPH. Mixing reduced *Bs*FNR and *Rp*FNR with NADP$^+$ resulted in a rapid formation of CT complexes followed by the oxidation of FNRs. In contrast, mixing reduced *Ct*FNR with NADP$^+$ provided no obvious absorption changes in CT band region ahead of its slow oxidation. Kinetic analyses of the reactions catalysed by *Bs*FNR and *Rp*FNR indicated that the rate-limiting steps were the hydride-transfer process in both directions. The hydride transfer rate for NADPH-oxidation direction was much faster than that for NADP$^+$ reduction direction in the case of *Bs*FNR, whereas those are comparable in the case of *Rp*FNR. In the case of *Ct*FNR, the reaction between *Ct*FNR and NADP$^+$/NADPH was reversible, in accordance with its physiological function, though the rate-limiting step in NADP$^+$ reduction was uncertain and the observed rate was too slow.

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Identification of inhibitor of UDP-galactopyranose mutase from *Aspergillus fumigatus*

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Keywords: flavin-dependent enzymes, non-redox chemistry, aspergillus

Aspergillus fumigatus is an opportunistic human pathogen and the most common causative agents of allergic bronchopulmonary aspergillosis and invasive pulmonary aspergillosis. Although there are treatments for *A. fumigatus* infections, the mortality rate among immunocompromised patients is >50%. The fungal cell wall is an essential organelle required to maintain the cell integrity. It also plays an important role in primary interactions between pathogenic fungi and their hosts since it provides adhesive properties and protection against host defense mechanisms. Galactomannan and galactofuranose are the major components of the A. FUMIGATUS cell wall. The enzyme UDP-galactopyranose mutase (UGM; E.C. 5.4.99.9) is a key enzyme the biosynthesis of galactofuranose. UGM is a flavoenzyme that catalyzes the conversion of UDP-galactopyranose to UDP-galactofuranose. Deletion of UGM in *A. fumigatus* causes an attenuation of virulence in a mouse model of invasive aspergillosis. Furthermore, the absence of galactofuranose results in a thinner cell wall that correlates with an increased susceptibility to several antifungal agents (1). The absence of human homologs of UGM and its importance in infection of *A. fumigatus* and human parasites such as *Leishmania major* (2) and *Brugia malayi* (3) makes it an attractive target for drug development. In this work, we present the results of a HTS assay using the previously reported ADP-TAMRA[4] fluoresce probe with a 2320 compounds library against UGM. Hit validation was performed with a series of orthogonal assays including UPLC and DSF. Natural occurring flavonoids were identified as inhibitors of AfUGM. In order to get an insight into the mechanism of inhibition, several derivatives of were also studied.

Research reported here was supported by the National Institute of General Medical Science of the National Institutes of Health under award number R01GM094469

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The optical absorption spectrum of reduced flavin is more complex than we knew: why it matters

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Keywords: photobiology, spectroscopy, Stark spectroscopy, TD-DFT

Chromophoric biomolecules are exploited as reporters of a diverse set of phenomena, acting as internal distance monitors, environment and redox sensors, and endogenous imaging probes. In the case of flavins, photoinduced electron transfer and photochemistry are also important outcomes of the absorption of light. In either case, it is of the greatest importance to have a precise knowledge of the direction(s) of the absorption transition dipole moment(s) (TDMs) in the molecular frame of reference for the absorption band in question. The directions of the TDMs in oxidized and semiquinone flavins were characterized decades ago,[1] and the details of charge redistribution in these forms have also been studied by Stark spectroscopy.[2] The electronic structure of the fully reduced hydroquinone anionic state, FlH^-, however, has been the subject of untested estimates of the number and direction of TDMs therein, as well the electronic structure changes that occur upon light absorption. Here we have used Stark spectroscopy to measure the magnitude and direction of charge redistribution in FlH^- upon optical excitation. These data were analyzed using TD-DFT calculations. The results show unequivocally that not one but two nearly orientation-degenerate electronic transitions are required to explain the 340–500 nm absorption spectral range, demolishing the commonly held assumption of a single transition. The difference dipole moments for these states show that electron density shifts toward the xylene ring for both transitions. These measurements force a reappraisal of previous studies that have used erroneous assumptions and unsubstantiated estimates of these quantities. The results put future optical studies of reduced flavins/flavoproteins on a firm photophysical footing.

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Stabilization of a cancer associated variant of human NQO1 by the use of small-molecular chaperones

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Keywords: single nucleotide polymorphism, cancer, chemotherapy, pharmacological chaperones

NAD(P)H:quinone oxidoreductase 1 (NQO1; EC 1.6.99.2) is a human FAD dependent enzyme catalyzing the two-electron reduction of quinones to hydroquinones. NQO1, for instance, plays an important role in the antioxidant defense system where it lowers the quinone levels and thereby prevents the formation of reactive oxygen species (ROS). Furthermore, it binds to the 20*S* proteasome, thus stabilizing several tumor suppressors like $p33^{ING1b}$, p53 and p73. [1, 2] The level of NQO1 is also increased in several tumors, therefore it is an important target for anti-cancer prodrugs.[3]

A naturally occurring single nucleotide polymorphism (*NQO1*2*) in the *NQO1* gene, results in the replacement of proline 187 to serine (P187S) in the protein sequence. The distribution of the homozygous *NQO1*2* among the population varies between 2 and 20% depending on the ethnical background. The reduced activity and stability of the NQO1 P187S variant leads to an increased toxicity of benzene, higher risk for several types of cancers and poor survival rate after anthracycline-based chemotherapy.[4]

A previous study gave insights into the structural characteristics of the NQO1 P187S variant and concluded that the single amino acid exchange destabilizes interactions between the core and C-terminal domain of the variant protein in solution.[5] The ongoing studies involve further investigations of the behavior of the NQO1 P187S variant as well as the possibilities to find a way to restore the activity and stability of the variant by small molecules. The first results show that the investigated compound binds to and stabilizes the NQO1 P187S variant.

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A phylogenetic analysis reveals various classes of fungal FAD-dependent glucose oxidoreductases

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Keywords: Phylogeny, Oxygen Reactivity, Glucose Oxidase, Glucose Dehydrogenase

Fungal FAD dependent oxidoreductases that act on carbohydrates have been experiencing continuous attention for their application in biofuel cells and biosensors since their introduction in 1962[1]. The two very closely related enzymes glucose oxidase (GOx) and glucose dehydrogenase (GDH) are the most commonly used enzymes in biosensors for glucose monitoring. Their main difference is the ability to use oxygen as an electron acceptor which is only attributed to GOx. Although these enzymes are already extensively used, the number of publications describing detailed characterization of GOx and GDH is still limited[2]. In this phylogenetic study we present a comprehensive overview of the GOx/GDH clade and the diversity in sequences it holds. By comparing the sequence alignment with published crystal structures[3,4], putative novel features of still uncharacterized enzymes could be identified. Based on this extensive sequence comparison containing over 250 sequences we propose a new classification for fungal GDH enzymes. Additionally we were able to identify features that are uniquely found in GOx enzymes and may contribute to the regulation of oxygen reactivity. The phylogenetic study will be further extended by ancestral sequence reconstruction and site directed mutagenesis to find the origin of oxygen reactivity in the clade of FAD-dependent glucose oxidoreductases. Furthermore, a variety of sequences covering all predicted structural differences and phylogenetic clades will be expressed and characterized to verify our computational predictions. Overall, we want to provide an overview of the naturally occurring variety of GOx and GDH enzymes in fungi.

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The role of valence electrons of FAD on the directivity of electron transfer in NADH-cytochrome b_5 reductase

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Keywords: FAD, X-ray crystallography, charge density analysis, hydrogen bond

Flavin in an oxidoreductive enzyme is a mediator of electron transfer. FAD in NADH-cytochrome b_5 reductase (b5R) transfers electrons from NADH to cytochrome b_5. The distribution of electrons on and around FAD is indispensable information for revealing the relationship between its function and structure in the protein molecule.

We performed the charge density analysis of b5R with X-ray crystallography at an ultra-high resolution of 0.78 Å[1]. Valence electrons on the FAD are clearly visualized as well as the peptide portion and assigned into the multipolar atomic model. The topological analysis for the determined electron density reveals the electronic structure of the isoalloxazine ring and hydrogen-bonding interactions in the protein environment. The tetrahedral electron distribution around the N5 atom of isoalloxazine is stabilized by hydrogen bonds with the surrounding peptide-backbone, $C_\alpha H$ of Tyr65 and amide-H of Thr66. The hydrogen bonding network leads to His49 composing the b_5-binding site via non-classical hydrogen bonds between N5 and $C_\alpha H$ of Tyr65 and $C_\beta H$ of His49. The improper electron transfer to the other direction are blocked by distorted peptide bonds. These results imply that the directivity of the electron transfer is realized by both of the nature of FAD itself and the protein environment.

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The structural biology of proline metabolism

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Keywords: proline dehydrogenase, protein structure, substrate channeling.

Proline metabolism has multifaceted roles in cancer, stress protection, protein chaperoning, cell signaling, programmed cell death, nutrient adaptation and survival, and pathogen virulence. I will present structural and functional data for some of the enzymes of proline metabolism, including the catabolic enzymes proline dehydrogenase (a flavoenzyme) and L-glutamate gamma-semialdehyde dehydrogenase (aka ALDH4A1), as well as the final enzyme of proline biosynthesis, pyrroline-5-carboxylate reductase (PYCR1). Of particular note, I will describe how the two catabolic enzymes communicate via substrate channeling.

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Engineering the regioselectivity of cyclododecanone monooxygenase

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Keywords: biocatalysis, protein engineering, Baeyer-Villiger oxidation, stereoselectivity.

Baeyer-Villiger monooxygenases (BVMOs) offer an attractive alternative to the traditional chemical Baeyer-Villiger oxidation. The biocatalyst uses molecular oxygen instead of peracids for the oxidation of substrates and, for asymmetric compounds, can result in the formation of preferred regioisomers. Interestingly, enzymes also do not strictly follow the purely chemical rationale for product distribution determined by the migratory aptitude, instead having the ability to modulate the reactivity in favor of the chemically less favorable product. The mechanism(s) by which this is accomplished are not well understood. Based on previous structural and kinetic studies of BVMOs, residues in or near the active site are thought to be responsible for the observed functional changes.

As part of our ongoing studies of BVMOs, we have explored changes to the regioselectivity of cyclododecanone monooxygenase from *Rhodococcus ruber SC1* through a combination of substitution experiments, probing the functional role of the enzyme-bound flavin cofactor and selected amino acid residues lining the substrate binding pocket in the active site. Our experiments demonstrate the significant impact of amino acid changes near the presumptive substrate binding pocket on the enzyme's enantio and regioselectivity for selected substrates including N-protected β-amino ketones.

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Styrene monooxygenase driven indole (derivative) monooxygenation for indigoid dye production

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Keywords: selective oxygenation, dye production, extinction coefficient, indigo

A number of oxygenases convert indole and derivatives to indigoid dyes such as Baeyer-Villiger monooxygenases, P450 monooxygenases, naphthalene dioxygenases, and styrene monooxygenases among others. Styrene monooxygenases (SMOs; EC: 1.14.14.11) are two-component flavoprotein monooxygenases allowing to selectively monooxygenate a number of substrates as styrene, indole, thioanisole, aliphatic alkenes and respective derivatives (halogenated, methylated, hydroxylated, hetero-aromatics) among others [1,2]. Interestingly, SMOs convert indoles only to a single product and no byproducts.

Three styrene monooxygenases (only oxygenase subunits originating of various bacteria) were recombinantly produced, purified to homogeneity and applied to screen indole conversion for a subsequent indigo production application. Therefore, the nicotinamide mimic 1-benzyl-1,4-dihydro-nicotinamide (BNAH) was applied as electron donor and this allowed reducing FAD in solution. Subsequently the reduced FAD was employed by the monooxygenase subunits to oxygenate indole (derivatives). Optimal concentration of BNAH for biotransformation as well as alternative electron donors had been determined.

Indigo formation rates (20 to 85 mU mg-1) in dependence of the applied monooxygenase (*Gp*StyA, *Vp*StyA1 or *Ro*StyA1) as well as of the provided substrate (indole, 6-fluoro indole, 6-chloro indole, or 6-bromo indole) were determined. Up-scaled production (10 mg substrate in 25 ml, 60 min, 30°C) by means of selected combinations was performed and allowed a 100% substrate conversion (e.g. *Gp*StyA, BNAH and 6-bromo indole). This allowed determining extinction coefficients of the pure indigoid dyes. Those have not been reported for halogenated indigoid dyes, yet. This furthermore, allowed quantifying enzyme activity and production rates.

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The origin of the berberine bridge enzyme-like protein family in plants

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Keywords: BBE-like, bicovalent flavoenzyme, plants, *Physcomitrella patens*

Berberine bridge enzyme (BBE)-like proteins form a large enzyme family found in bacteria, fungi and plants. In all these kingdoms of life these enzymes were shown to catalyze oxidation reactions in various metabolic pathways. While some BBE-like proteins mediate the simple oxidation of alcohols to aldehydes, others were found to be involved in more complex processes such as ring closing reactions. Even though the type of reactions these proteins are catalyzing may be very diverse, the enzyme family shares some common features. The mostly bicovalent attachment of the flavin cofactor strongly determines their catalytic properties, since this special type of flavin tethering enhances the oxidative power of the cofactor and at the same time opens the active site for larger substrates[1, 2].

During evolution of the plant kingdom not only the diversity of the reactions catalyzed by BBE-like proteins was enlarged, but also the number of *bbe*-like genes per species strongly increased. The first land plants, such as the moss *Physcomitrella patens*, have coding sequences for just one or two BBE-like protein(s), while higher developed species possess up to 65 *bbe*-like genes (western poplar: *Populus trichocarpa*). Until now, the function of most of these BBE-like enzymes is unknown, but their great diversity motivates researchers to deepen their understanding of this protein family.

To gain insight into the development of the enzyme family especially studies on enzymes from lower plants would be of great advantage. Therefore, we initiated the biochemical characterization of the heterologously expressed enzyme from *Physcomitrella patens*, with the main focus set on the substrate specificity and the catalytic mechanism of the enzyme.

To be able to tell more about the importance of the gene for the native host, also *in vivo* studies with a *Pp*BBE knock-out strain are currently conducted.

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Kinetics and product analysis of oxidase and monooxygenase activities of L-amino acid oxidase and monooxygenase

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Keywords: L-Amino acid oxidase/monooxygenase, L-Lysine α-oxidase, L-Lysine monooxygenase, Flavin monooxygenase, Flavin oxidase

L-Amino acid oxidase/monooxyganase (L-AAO/MOG) from *Pseudomonas* sp. AIU 813 is a non-covalent FAD-bound enzyme that catalyzes mixed activities of an oxidative decarboxylation as well as an oxidative deamination of L-amino acids. The enzyme uses L-lysine as a native substrate and oxygen is used as an electron acceptor. L-AAO/MOG has potential to be used as enzyme sensor for detection of L-lysine by measuring the amount of H_2O_2 formed. The reaction of the oxidase (deaminase) path yields α-keto acid, hydrogen peroxide and ammonia as products while amide and carbon dioxide are formed by the monooxygenase (decarboxylase) path. The wild-type enzyme can only use L-lysine, L-arginine and L-ornithine, not other L- or D-amino acids as substrates. We have investigated the catalytic features of L-AAO/MOG that control these two activities of the enzyme. Transient kinetics of the L-AAO/MOG reactions was studied using stopped-flow spectrophotometry. For flavin reduction, rates of the flavin reduction depend on L-lysine concentration. Charge-transfer complex of the enzyme with L-Lysine is formed during the flavin reduction. For flavin oxidation, the reactions showed two and three phases kinetics when dithionite and L-lysine were used for preparing the reduced enzyme, respectively. No C4a-hydroperoxyflavin was detected and rates of the flavin oxidation depend on O_2 concentration. However, variation of L-lysine concentration does not affect the rate of the third phase. Product analysis of multiple turnover reactions of L-lysine and L-ornithine were carried out by HPLC/MS. Results show that for L-ornithine reaction, no amide product was formed in monooxygenase path. On the other hand, it shows a decarboxylated product from α-keto acid that is different from L-lysine reaction.

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Human MICAL-1 the multidomain flavoenzyme participating to actin cytoskeleton dynamics

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Keywords: MICAL, oxidase/monooxygenase, F-actin depolymerization, oxidative stress.

MICAL (from the Molecule interacting with CasL) indicates a family of multidomain proteins conserved from insects to humans, which are increasingly attracting attention for their participation in the control of actin cytoskeleton dynamics, and, therefore, in the several related key processes in health and disease. MICAL is unique among actin binding proteins because it catalyzes a NADPH-dependent F-actin depolymerizing reaction. This unprecedented reaction is associated with its N-terminal FAD-containing domain that is structurally related to p-hydroxybenzoate hydroxylase, the prototype of aromatic monooxygenases, but catalyzes a strong NADPH oxidase activity in the free state. The additional calponin homology (CH), LIM (from Lin-11, Isl-1 and Mec-3 gene products) and C-terminal regions, which are typical protein interaction domain, are believed to play modulatory roles. In order to contribute to the understanding of this class of important enzymes we have produced full-length human MICAL-1 and truncated forms progressively lacking the C-terminal region, the LIM and also the CH domain1-3. From their comparison it is emerging that the MICAL-1 NADPH oxidase activity may contribute to its biological role and it is also responsible of the F-actin depolymerizing activity, as opposed to the hypothesis that MICAL switches from an oxidase (in the free state) to a specific actin methionine hydroxylase (when F-actin bound). The CH and LIM domains increase the K_m for NADPH of the free enzyme but have no effect on the reaction in the presence of F-actin. The C-terminal region is responsible of the MICAL-1 autoinhibition, which is part of the biological control mechanism. The equilibrium between the active and active MICAL conformations in solution is being studied by combining kinetics and structural approaches also using MICAL interactors.

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MonoX, a flavoprotein with enigmatic function

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Keywords: flavoprotein, genome mining, monooxygenase, *Rhodococcus opacus* 1CP

Flavoprotein hydroxylases constitute group A of the family of flavoprotein monooxygenases (1,2). Genome mining of *Rhodococcus* species revealed that these actinomycetes contain many group A members with unknown function. Here, we report the cloning and biochemical characterization of MonoX from *Rhodococcus opacus* 1CP. MonoX is highly expressed in *E. coli* and purified using a C-terminal His_6-tag. MonoX is a monomer of 44 kDa, which tightly binds FAD.

Homology modeling showed that MonoX has structural features in common with salicylate hydroxylase (2-hydroxybenzoate 1-hydroxylase: decarboxylating), 6-hydroxynicotinate 3-monooxygenase and 3-hydroxybenzoate 6-hydroxylase. However, its active site shows a remarkable absence of histidine and tyrosine residues present in these enzymes, instead it contains more hydrophobic residues.

Activity assays with 70 candidate substrates revealed that MonoX displays NADH oxidase activity with several salicylate analogues. However, with none of these effector molecules product formation was observed. Gene cluster analysis neither did give a clue to its 'true' substrate. Close inspection of the active site of modeled MonoX indicated that the position of a substrate in MonoX must deviate from that as found in salicylate hydroxylase and 6-hydroxynicotinate 3-monooxygenase. A MonoX crystal structure and advanced docking might shed more light on the elusive nature of compound X.

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A light-driven cascade to promote peroxygenase catalyzed reactions

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Keywords: biocatalysis, photochemistry, regeneration system, oxidation

The oxyfunctionalization of non-activated C-H bonds is one of the greatest challenges in organic chemistry [1,2]. Peroxygenases are promising catalysts that can achieve selective hydroxylation of non-activated C-H bonds using hydrogen peroxide (H_2O_2) as oxidant. However, these enzymes exhibit poor robustness against the latter. Therefore, methods for the in situ supply with H_2O_2 have to be investigated. Most commonly, this is achieved by catalytic reduction of molecular oxygen [3,4].

One promising approach may be to use formate as a sacrificial electron donor to promote the reductive activation of O_2. Here, we propose a photochemoenzymatic method comprising formate dehydrogenase and flavins under visible light irradiation (Figure 1). In this approach, H_2O_2 is produced by the spontaneous reduction of O_2 by a reduced flavin (FMN_{red}). This flavin (FMN_{ox}) is then regenerated by an photochemoenzymatic system. The photoexcitation of FMN_{ox} by visible light enables its reduction by NADH which is regenerated by the consumption of formate by formate dehydrogenase (FDH).

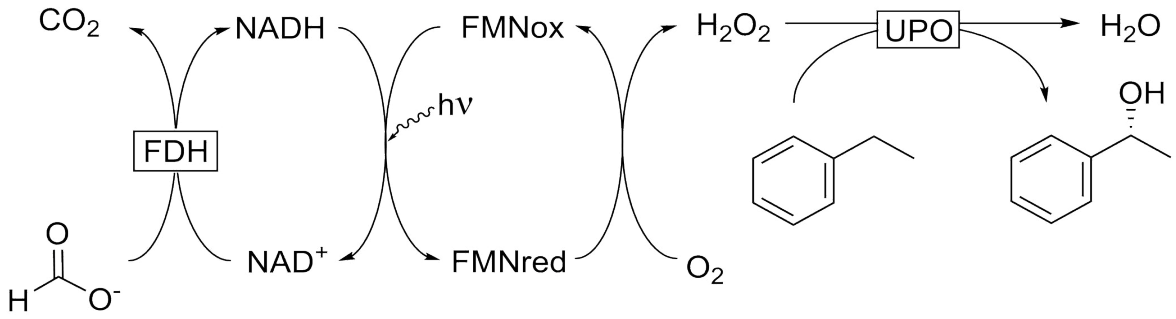

Figure 1. Photochemoenzymatic generation of H_2O_2 to promote Unspecified Peroxygenase (UPO) catalyzed hydroxylation.

The setup and characterization of this novel approach will be discussed.

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Molecular mechanisms of light regulation in LOV-diguanylate cyclases - implications for sensor-effector modularity.

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Keywords: photoreceptor, flavin, GGDEF, modularity

The ability to respond to environmental cues is a prerequisite for homeostasis of all organisms. One important external stimulus is light and nature has evolved a variety of sensory modules that allow the integration of different light qualities and quantities. One frequently occurring class of photoreceptor modules are flavin-bound blue light sensing domains that are found in all kingdoms of life.

Among those, LOV domains (sensors of Light, Oxygen and Voltage) have attracted special attention due to their modularity in regulating a variety of effector domains. This property has been exploited for the generation of various artificial light-regulated functionalities with applications in the field of optogenetics1. While this might suggest that principles of sensor-effector coupling are well understood, the generation of such tools still requires substantial screening efforts and molecular details of light-regulation are not known in most cases.

In order to better understand the concept of light signal integration in sensor-effector systems, we are focusing on diguanylate cyclases (GGDEF domains) fused to LOV domains and other sensory modules. Due to the relatively strict requirements in linking various sensors to GGDEF domains2, our functional and structural characterization of these systems revealed important insight into the naturally occurring modularity of sensor and effector domains. We will present details of the biochemical and functional characterization together with a structural analysis of a full length LOV-regulated diguanylate cyclase. In addition, we will also compare these novel insights with a recently characterized red-light regulated diguanylate cyclase3, which allows us to identify common mechanisms of signal integration in diguanylate cyclase systems and provides new insight into the astonishing modularity of naturally occurring sensor-effector couples.

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The sugar oxidation of pyranose 2-oxidase

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Keywords: Pyranose 2-oxidase, Glucose-Methanol-Choline (GMC) oxidoreductase superfamily, flavin adenine dinucleotide (FAD), density functional theory

Pyranose 2-oxidase catalyzes the oxidation of aldopyranoses by using molecular oxygen as an electron acceptor to yield the corresponding keto-aldoses and hydrogen peroxide. This enzyme belongs to the Glucose-Methanol-Choline (GMC) oxidoreductase superfamily and contains flavin adenine dinucleotide (FAD) as a cofactor. P2O catalyzes regio-specific oxidation at the C2 position. Here, the hydride transfer reaction from glucose to oxidized flavin catalyzed by pyranose 2-oxidase (P2O) was investigated by density functional theory calculations and transient kinetics. Our findings suggest that the first step of the P2O reaction is a hydride transfer from C2 position of glucose to N5 of the flavin. Then, the proton abstraction occurs by the conserved residue, His548. In fact, the hydride transfer enhances the proton acceptor ability of His548. The computational results are consistent with kinetic studies of variant forms of P2O at residues His167, Thr169, Val546, His548, and Asn593, and kinetic isotope effects and pH-dependence studies of the wild-type enzyme. The interactions around the sugar binding site (Thr169, Gln448, Asp452, Tyr456, Phe474, Val546, His548, and Asn593) are important for dictating the formation of the carbocation intermediate. Our findings also suggest that P2O can convert not only monosaccharides (glucose, galactose, xylose, arabinose, and mannose) but also disaccharides (maltose and sucrose). Therefore, the enzyme is useful for providing a pool of keto-sugar intermediates for synthesis of rare sugars, fine chemicals and drugs. Knowledge obtained from these studies should be useful for industrial applications to produce high value sugars.

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Signal activation in LOV domain proteins: N5 nitrogen protonation and internal electron transfer

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Keywords: LOV domain, photoreceptor, neutral semiquinone, methionine residues

Blue light excitation of the canonical light-oxygen-voltage (LOV) domain leads to thioether bond formation between the C4a carbon of the isoalloxazine ring and a conserved cysteine residue. Adduct formation results in N5 nitrogen protonation and changes in the hydrogen bond network surrounding the cofactor, instigating protein conformational changes and downstream signaling. Interestingly, when the circadian-clock LOV-protein Vivid from *Neurospora crassa* is devoid of the adduct-forming cysteine (VVD-III), the neutral semiquinone (NSQ) forms upon photoreduction. This variant is surprisingly capable of dimerization and *in vivo* signaling.[1] Moreover, analogous LOV domains of natural cysteine-less proteins were discovered to exhibit conformational changes after either chemical reduction or photoreduction, suggesting the NSQ is a biologically functional state.[1] In an effort to engineer a null variant of VVD-III, all tyrosines were changed to phenylalanines, yet the protein still readily undergoes photoreduction. However, replacing the methionine residues with selenomethionine residues produced substantial changes in photoreduction properties. Absorption, fluorescence, and infrared spectroscopies of various VVD mutants signify that electron transfer most likely occurs via the methionine residues. Overall, our findings implicate that 1) flavin N5 protonation is sufficient for triggering signal transduction, and 2) internal methionine residues can act as effective electron donors to high potential cofactors in proteins.

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Screening of the effectors for human D-amino acid oxidase and the analyses of structure-activity relationships

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Keywords: D-amino acid oxidase, catechins, screening of inhibitor, molecular docking

D-Amino acid oxidase (DAO) degrades D-type amino acids by the oxidative deamination in kidney, liver and brain. D-Serine, one of the substrates of DAO, is the co-agonist of NMDA receptors, whose activation and inactivation correlate to neuronal degenerative and psychiatric disorders, respectively. Therefore, the searching the effectors, both activators and inhibitors, for human DAO will lead to new therapeutic medication for neurological diseases.

Screening the effectors of DAO was performed with 1593 compounds in the drug-seed library of Tokushima university by the direct assay where the initial rate of the reduction of redox dye DCPIP was monitored in the presence of D-proline and human DAO. Twelve compounds accelerated the assay reaction, but all of them were either the oxygen scavengers or the direct reactants to DCPIP. On the other hands, we identified twenty-four compounds as the candidates for inhibitor. They are comprised of carboxylic acid derivatives, heterocyclic compounds, halogenated compounds, and polyphenol catechins, and the kinetic analysis confirmed that all these compounds are the competitive inhibitors to substrate D-proline. Among these inhibitors, the condensed-catechins exhibited strong inhibition, although the chemical structure are different from the substrate D-amino acids by lacking the functional group of carboxylic acid.

QSAR analyses of six stronger inhibitory catechins suggested that the inhibition of DAO correlated well with molecular mass, hydrophobicity, ability of the hydrogen bonding and the flexibility of conformations. Molecular docking analysis of nineteen catechins from the library revealed three binding sites for the catechin molecules at the cavities on the surface of DAO, and only one site located at the entrance of the active site pocket is the potential inhibitory binding site for these condensed catechins.

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X-ray structure analysis of fructosyl peptide oxidases to elucidate the residues responsible for the oxidative half-reaction

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Keywords: X-ray structure, fructosyl peptide oxidase, protein channel, *Phaeosphaeria nodorum*

FAD-containing fructosyl amine oxidases (FAOXs) catalyze the oxidative deglycosylation of fructosyl amino acids such as fructosyl valine and/or ε-fructosyl lysine, which are the degradation products of glycated hemoglobin (HbA1c) and glycated albumin (GA), respectively. As members of the FAOX family, fructosyl peptide oxidases (FPOXs) are also known as candidates for diagnostic enzymes. We reported on a novel FPOX from *Phaeosphaeria nodorum* (PnFPOX)[1]. PnFPOX showed a remarkably low *K*m value for f-$^{\alpha}$Val-His (0.185 mM), distinguishing it from previously reported FPOXs. In the study on PnFPOX, an engineered PnFPOX, the mutant Asn56Ala (N56A) PnFPOX showed lower oxidase activity when oxygen was used as the electron acceptor, while its dye-mediated dehydrogenase activity was higher than that of wild-type[2]. Therefore, elucidation of the structures of the wild-type and Asn56Ala mutant PnFPOX will be promising to provide significant information needed to understand the oxidative half-reaction of this enzyme group.

In this study, we determined X-ray structures of wild-type and N56A mutant PnFPOX. Comparison of the structures at the *si*-face of FAD in PnFPOXs showed an intriguing difference between the wild-type and N56A. In wild-type PnFPOX, Asn56 forms a hydrogen bond with Lys274, thereby preventing it from forming a salt bridge with Asp54. By contrast, Lys274 of PnFPOX N56A moves toward Asp54, and they approach each other to form a salt bridge. Site-directed mutagenesis studies and protein channel analysis suggest that Asp54 assists in accepting oxygen properly at the position of the bound water molecule in the main oxygen channel. These results reveal that Asn56 in PnFPOX is essential for maintaining an effective oxygen accession path, and support the role of Asp54 as a gate keeper that cooperates with Lys274 to enable oxygen to reach the active site properly.

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Synthesis of new deazaflavins as mediators for new biocatalytic processes

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Keywords: deazaflavins, mediators, biocatalytic processes

Biocatalysts are gaining ground as competitive tools for organic chemistry.1 Their high selectivity enables shorter and more efficient synthetic routes and bears the promise for greener chemistry. Recently, our group described a light-driven direct cofactor regeneration system for the flavin-dependent Baeyer–Villiger monooxygenase PAMO and the enoate-reductase YqjM, deriving reducing equivalents from the inexpensive sacrificial electron donor EDTA.2,3 Also, Hollmann et al. reported the first light-driven deazaflavin-dependent direct enzyme regeneration system for a P450-BM3 catalyzed CH-activating hydroxylation.4

A range of new deazaflavins were prepared and characterized. Understanding of the mechanism of deazaflavin-dependent regeneration and use of deazaflavin derivatives in new biocatalytic reactions will be presented and discussed.

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Structural basis of kynurenine 3-monooxygenase inhibition

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Keywords: Kynurenine 3-monooxygenase, Kynurenine pathway, inhibition, virtual screening.

Kynurenine 3-monooxygenase (KMO) is an NADPH-dependent flavin monooxygenase, which catalyses the hydroxylation of the L-tryptophan metabolite L-kynurenine to form 3-hydroxykynurenine. It is a key enzyme in the kynurenine pathway (KP), the major catabolic route of tryptophan, and lies at a critical branching point of the KP, which makes KMO an attractive drug target for several neurodegenerative and neuroinflammatory diseases. Pharmacological benefits have been observed with therapeutic administration of KMO inhibitors in several disease models1, 2, but the fact that all inhibitors to date are unable to cross the blood brain barrier has prevented further drug development. We have used a combination of steady state and stopped flow kinetics to determine the reaction mechanism of KMO with selected inhibitors. This has facilitated more detailed insights into the mode of inhibition by the identified KMO inhibitors that might assist future KMO inhibitor design.

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Index