Symposium on Biocatalysis
Tuesday, November 5th 2013

at the Max Planck Institut für Kohlenforschung,
Lembkestraße 7, 45470 Mülheim an der Ruhr, Grand Lecture Hall

Program
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Welcome to the
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Mülheim an der Ruhr, Tuesday, November 5th 2013

Dear guests,

The Max Planck Institut für Kohlenforschung, founded in 1912 as the Kaiser Wilhelm Institut für Kohlenforschung, pursues basic research in chemistry and in particular in all fields of catalysis. The central theme pervading all Departments is basic research in the catalytic transformation of compounds and materials with the highest degree of chemo-, regio- and stereoselectivity under conditions which maximize efficient use of natural resources.

Biocatalysis has been a very important aspect of the research at our institute and was introduced here with the pioneering studies of Manfred Reetz shortly after his arrival in Mülheim in 1991. His research involves the introduction of molecular biology into synthetic organic chemistry in the quest to exploit directed evolution of stereoselective enzymes.

Manfred Reetz, who celebrated his 70th birthday this year, has influenced the development of our institute decisively. We are all very thankful for his commitment.

Dear colleagues, we are all very happy to welcome you at our institute and we are very excited to attend today’s lectures.

Ben List
Agenda

08:30  Doors Open, Coffee (Foyer of the Institute, 1st floor)
09:20  Welcome, Ben List
09:30  Introduction, Manfred Reetz
09:45  Karl-Erich Jäger (Jülich/Düsseldorf):
       “What lipases teach us and we teach lipases”
10:30  Don Hilvert (Zürich):
       “Building Better Enzymes”
11:15  Coffee Break (Foyer of the Institute, 1st floor)
11:30  Michael Müller (Freiburg):
       “Unprecedented Role of Hydronaphthoquinone Tautomers in Biocatalysis”
12:15  Uwe Bornscheuer (Greifswald):
       “Modern Tools for Protein Engineering and Enzyme Cascade Reactions”
13:00  Lunch (Foyer of the Institute, Ground Floor)
14:30  Nicholas Turner (Manchester):
       “Design and Evolution of New Biocatalysts for Organic Synthesis”
15:15  Ulrich Schwaneberg (Aachen):
       “Protein Engineering for Biocatalysis and Interactive Materials”
16:00  Coffee Break (Foyer of the Institute, 1st floor)
16:15  Michael Bott (Jülich):
       “Single-cell metabolite sensors and recombineering as novel tools for strain
       and enzyme development”
17:00  Frank Schulz (Dortmund):
       “Engineering Multistep Biocatalytic Cascades in vivo:
       Chemo-microbial synthesis of natural product derivatives”
17:45  Christian Hertweck (Jena):
       “Decoding and Harnessing Complex Microbial Assembly Lines”
18:30  Manfred Reetz
       Final Remarks
19:00  Reception (Foyer of the Institute, Ground Floor)
What lipases teach us and we teach lipases

Karl-Erich Jaeger

Lipases are natural fat-splitting enzymes occurring in microorganisms, plants, animals and humans. Due to their unique properties they have found numerous industrial applications, e.g. as detergent additives, in the production of food ingredients, biodiesel and, last not least, as biocatalysts for the synthesis of therapeutics. Most of these industrial relevant lipases originate from microbes with the fungal lipase CALB from *Candida antarctica* (recently renamed *Pseudozyma antarctica*) and the bacterial lipase PAL from *Pseudomonas aeruginosa* being prominent examples. More than a decade ago, PAL served as a model enzyme to demonstrate that directed evolution can be used to create enantioselective enzymes [1, 2] and meanwhile, PAL became the most thoroughly studied bacterial enzyme with respect to evolving substrate scope and enantioselectivity. Our initial work which has been carried out during a long and fruitful collaboration with the group of Manfred Reetz will briefly be reviewed. Recently, we have studied other industrial relevant lipases including LipA from *Burkholderia glumae* which is used by BASF SA for the production of enantiopure alcohols and amines. Here, we have investigated the global physiological response of this bacterium to lipase overexpression using genomics, transcriptomics, proteomics, and metabolomics. Another study was carried out to elucidate the structural prerequisites of lipase temperature stability. Here, we have comparatively expressed, purified, and biochemically characterized three lipases originating from a psychrophilic, a mesophilic and a thermophilic bacterium, respectively. Our results allowed us to identify a defined loop structure which significantly affects thermal stability. More recently, we have identified and characterized novel flavin-based fluorescent proteins (FbFPs) derived from plant and bacterial light, oxygen, voltage (LOV) photoreceptors. Fusions of FbFPs to a bacterial lipase and a plant lyase were constructed and analyzed demonstrating the high biotechnological potential of such light-responsive biocatalysts [3, 4].

References
Building Better Enzymes

Donald Hilvert

Enzyme design represents a formidable challenge. Extensive mechanistic and structural studies have provided a solid qualitative understanding of enzyme action. Nevertheless, our knowledge of structure-function relationships in these macromolecules remains incomplete and a quantitative accounting of the incredible efficiency achieved by enzymes still eludes us. Diverse strategies have therefore been explored to engineer enzymes for novel applications, ranging from repurposing existing active sites to generation of antibodies with tailored catalytic properties. Among these approaches, computational design has emerged as particularly promising.\(^1\,\text{2}\) Computational enzyme design has afforded made-to-order catalysts for a variety of reactions lacking biological counterparts, including simple proton transfer reactions, multi-step retroaldol transformations, Diels-Alder cycloadditions, and several metal-dependent processes. Although the starting activities of these artificial enzymes are typically low, they can be significantly increased by directed evolution. In favorable cases, activities approaching those of natural enzymes have been achieved.\(^3\) Analysis of the (sometimes surprising) evolutionary trajectories provides valuable feedback for the design process as well as insights into natural protein evolution. Recent progress on the computational design and evolutionary optimization of artificial enzymes will be surveyed in this lecture, highlighting both the opportunities and challenges facing this emerging field.

\(^1\) H. Kries et al., *Curr. Opin. Chem. Biol.* 2013, 17, 221-228


Unprecedented Role of Hydronaphthoquinone Tautomers in Biocatalysis

Syed Masood Husain, Michael Schätzle, Michael Richter, Steffen Lüdeke, Michael Müller

The formation of fungal DHN melanin is not a straightforward pathway, but rather a complex network. Many metabolites have been shown to be derived from intermediate polyhydroxynaphthalenes. 4-Hydroxy-1-tetralones and 3,4-dihydroxy-1-tetralones observed in the melanin pathway have been proposed to be the products of oxidation of the respective polyhydroxynaphthalenes to the naphthoquinones, followed by a double reduction via the hydronaphthoquinones.

In this context, we recently reported the enzymatic formation of 3,4-dihydroxy-1-tetralones through reduction of 2-hydroxy-1,4-naphthoquinones by NADPH-dependent tetrahydroxynaphthalene reductase (T4HNR). To elucidate the mechanism of these transformations, we used lawsone (1) as a model substrate. We found that the unprecedented mechanism of the formation of the vicinal ketodiol 2 from 1 proceeds via the formation and reduction of the nonaromatic 1,4-diketo tautomer 3 of hydronaphthoquinone 4 (Scheme).

Our results also shed light on the formation of 4-hydroxy-1-tetralones and provide evidence for a more essential role of hydroquinone tautomers in biocatalysis and probably biosynthesis.

Modern Tools for Protein Engineering and Enzyme Cascade Reactions

Uwe T. Bornscheuer

Protein engineering has developed in the past decade to a highly important technology\textsuperscript{1,2} as it is a useful tool to create enzymes with desired properties (with respect to e.g., substrate specificity, stereoselectivity or thermostability), but also helps to understand how proteins evolved and how they function.

Whereas initially rational protein design based on detailed analysis of three-dimensional structures was the method of choice, directed evolution – in essence a random mutagenesis followed by screening or selection of desired mutants – became an important alternative. In this lecture, the principle strategies and current challenges in protein engineering will be highlighted. Examples will include the creation of an epoxide hydrolase from an esterase scaffold within the $\alpha/\beta$-hydrolase fold enzyme family\textsuperscript{3} and the inversion of enantioselectivity of an esterase active towards sterically demanding tertiary alcohols\textsuperscript{4}. In addition, we developed a method for in vivo selection and cell sorting to identify enantioselective enzymes.\textsuperscript{5} The vast number of protein sequences available from databases substantially facilitates protein engineering. We used this plethora of information to create ‘small, but smart’ focused protein libraries from the analysis of $>2.800$ sequences of enzymes from the $\alpha/\beta$-hydrolase fold family using the 3DM database\textsuperscript{6}. This resulted in enzyme variants with substantially enhanced thermostability, enantioselectivity and altered substrate range\textsuperscript{7}. For the synthesis of chiral amines, we developed \textit{in silico} analysis and identified a toolbox of novel ($R$)-selective transaminases\textsuperscript{8} as well as ($S$)-selective enzymes from a structure-guided search.\textsuperscript{9} More recently, we focused our interest enzyme cascade reactions. Examples include the conversion of unsaturated fatty acids to valuable $\omega$-hydrocarboxylic acids, dicarboxylic acids and alcohols.\textsuperscript{10} Furthermore, we could combine alcohol dehydrogenase, enoate reductase and monoxygenase to enable \textit{in vivo} biotransformations.\textsuperscript{11}

\textsuperscript{11} Oberleitner, N., Peters, C., Muschiol, J., Kadow, M., Saß, S., Bayer, T., Schaaf, P., Iqbal, N., Rudroff, F., Mihovilovic, M.D., submitted.
Design and Evolution of New Biocatalysts for Organic Synthesis

Nicholas J. Turner

This lecture will describe recent work from our laboratory aimed at developing new biocatalysts for enantioselective organic synthesis.\(^1\) For example, monoamine oxidases (MAO-N) are a family of enzymes that catalyze the (S)-selective oxidation of amines to imines. MAO-N can be used as biocatalysts to obtain enantiomerically pure chiral amines by deracemisation or desymmetrisation of substrates. Recently new variants of MAO-N have been developed via a combination of directed evolution and rational design in order to broaden the enzyme’s substrate specificity.\(^2\)\(^-\)\(^3\)

The new mutants have been used for the deracemisation of primary and secondary amines such as (R)-4-chlorobenzhydrylamine (building block for the synthesis of Levocetirizine), (S)-1-phenyl-1,2,3,4-tetrahydroisoquinoline (building block for the synthesis of Solifenacin) and the two alkaloids (R)-Harmicine and (R)-Eleagnine. We have also recently developed a new broad specificity (R)-selective amine oxidase based upon D-hydroxynicotine oxidase.

The integration of several biocatalytic transformations into multi-enzyme cascade systems has also been a focus of recent work in our laboratories. In this context MAO-N has been used in combination with other biocatalysts and chemocatalysts in order to complete a cascade of enzymatic reactions.\(^4\) In particular, a biocatalytic tandem reaction combining MAO-N and ATHase has been developed for the deracemisation of 1-methyl tetrahydroisoquinoline, nicotine and 2-substituted pyrrolidine and a combination of MAO-N and \(\omega\)-transaminases was employed for a one-pot synthesis of enantiopure 2,5-disubstituted pyrrolidines starting from different 1,4-diketones. We are also developing combined oxidase/metal-catalyst systems for the selective oxidation of amines and alcohols to amides/lactams.

Protein Engineering for Biocatalysis and Interactive Materials

Ulrich Schwaneberg

Protein engineering by directed evolution and semi-rational design have become widely applied strategies for tailoring enzyme properties to needs in biocatalysis\(^1\). Highlights comprising reengineered monooxygenases for ortho-selective hydroxylation of halobenzenes\(^2\), inversion of styrene epoxidation\(^3\), double oxidation of cyclic alkanes under cofactor regeneration\(^4\), and improvements in properties of hydrolases and oxidases (peracid formation, organic solvent/salt resistance\(^5\) and mediated electron transfer\(^6\)) will be presented in the first part.

In the second part a novel technology platform for catalysis based on reengineered proteins for controlled compound flux\(^8\) (\textit{E. coli} iron-transporter FhuA; ß-barrel protein) and anchoring peptides for monolayer decoration of fibers and surfaces will be introduced (cecropin example). The interactive materials research platform aims on the long run to develop chiral membranes and functional film coatings with a density of a couple of hundred thousands of reengineered FhuA channel proteins per cm\(^2\) ref.\(^9\). The presentation will be focused on the progress in respect to FhuA reengineering (triggers; hybrid catalysts) which is part of research agenda of the DWI.\(^{10}\)

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10 http://www.wissenschaftsrat.de/download/archiv/3185-13.pdf (Suchbegriff proteinengineering)
Single-cell metabolite sensors and recombineering as novel tools for strain and enzyme development

Michael Bott

Strain and enzyme development are key to the successful establishment of industrial bioprocesses. Rational strategies based on strain and enzyme engineering are highly successful, yet, random mutagenesis and screening remain an important alternative. The major bottleneck in the latter approach is the necessity to screen large libraries for those cells or enzymes showing the desired properties. Unfortunately, many metabolites of industrial interest do not have an easily detectable phenotype such as color and therefore screening by complex analytical methods is a laborious and time-consuming process. We developed an approach which can overcome this limitation. It allows the visualization of intracellular metabolite concentrations in single bacterial cells and the application of fluorescence-activated cell sorting (FACS), the highest-throughput method currently available. The approach is based on transcriptional regulators, which naturally detect a variety of ligands, but can also be engineered to detect the compound of interest.

In proof-of-concept studies, a single-cell L-lysine sensor for Corynebacterium glutamicum was established based on the transcriptional regulator LysG, which activates expression of the lysine exporter gene lysE when the cytoplasmic L-lysine concentration increases above regular levels [1]. In the sensor plasmid pSenLys, expression of eyfp encoding enhanced yellow fluorescent protein is controlled by LysG and thus by the intracellular L-lysine concentration. When pSenLys was transferred into a series of defined L-lysine producing strains, a linear relationship between specific fluorescence and cytoplasmic L-lysine concentration was detected in the range of 4 – 20 mM. The sensor was used to rapidly isolate by FACS hundreds of L-lysine producing cells from a large library of randomly mutagenized C. glutamicum wild-type cells [2]. By whole-genome sequencing of selected mutants, murE was identified as a novel target for improving lysine formation [2]. A major challenge of our approach is to test the effects on production of a large number of point mutations identified by whole-genome sequencing, which requires their targeted introduction into the genome. As current methods for C. glutamicum are very laborious and time-consuming, we established recombineering as an alternative and fast method using single-stranded oligonucleotides carrying the mutation of interest and a recombinase from E. coli prophage Rac [3]. The combination of recombineering and FACS-based screening (RecFACS) allows the rapid identification of productive point mutations [3].

LysG does not only respond to L-lysine, but also to L-arginine and L-histidine [1]. We made use of this property to screen plasmid libraries of aspartate kinase, N-acetylglutamate kinase and ATP phosphoribosyltransferase for variants with diminished feedback-inhibition by lysine and threonine, arginine, and histidine, respectively. Such variants were rapidly identified and the resulting strains produced significant amounts of L-lysine, L-arginine, or L-histidine [4]. Thus, our approach can be applied both genome- and enzyme-directed. Besides pSenLys, we meanwhile also constructed sensors for L-methionine and branched-chain amino acids [5], for L-serine and O-acetyl-serine, and further ones are in progress.

1 Bellmann et al. (2001) Microbiology 147: 1765-1774
2 Binder et al. (2012) Genome Biol 13: R40
4 Schendzielorz et al. (2013) ACS Synth Biol, in press
5 Mustafi et al. (2012) Metab Eng 14: 449-457
Engineering Multistep Biocatalytic Cascades in vivo: Chemo-microbial synthesis of natural product derivatives

Frank Schulz

Natural products are a rich source of chemical complexity and diversity, exemplified by their widespread application in medicinal chemistry. Of particular interest among natural products are polyketides and terpenoids, whose structural complexity poses long-standing challenges to chemists and are in the focus of our group’s research efforts.

In our work, the targeted manipulation of the biosynthetic enzyme machinery with enzyme sizes up to 4,000 kDa provides major tools to elucidate opportunities for the predictable and transferable manipulation of polyketide\(^1,2\) and terpene\(^3\) biosynthetic pathways. This is merged with organic synthesis to yield natural product derivatives.

For example, the focused synthesis of new polyketide derivatives through mechanism-based modifications of bacterial type 1 polyketide synthases will be explained.\(^1,2\)

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3 Arens et al., Chem. Commun. 2013, 49, 4337-4339.
Decoding and Harnessing Complex Microbial Assembly Lines

Christian Hertweck

Microorganisms are a prolific source for potential therapeutics and drug leads, in particular in the area of antiinfectives and antitumoral agents. From a synthetic point of view, it is fascinating to learn that the most complex metabolites are composed from simple biosynthetic building blocks by multienzyme assembly lines.

In recent years, advances in genome sequencing and developments of molecular tools have provided deeper insights into the genetic codes for these fine-tuned, multifaceted biocatalysts.

In this overview, I will demonstrate examples of using genetic and chemical methods in a synergistic manner to decode, study and alter biosynthetic processing lines in microorganisms.
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How to reach the Max Planck Institut für Kohlenforschung

By car

From the North (Duisburg, A3), the South (Köln, A3) or the West (Düsseldorf / Airport, A52): Leave highway at junction "Kreuz Breitscheid" in direction of Essen. Already in the junction take exit marked "Ratingen - Breitscheid" and continue on federal road B1 through Mülheim. After 8 km the road crosses the Ruhr river and winds up a hill. Near the top keep to the left, take a sharp left turn into Bismarckstraße (note the sign "Max-Planck-Institute"). Follow the third road to the right (there is another sign "Max-Planck-Institute") for 300 m to reach the Institute, entrance Lembkestraße.

From the East (Essen / Dortmund): Coming along on highway A40 you exit at "MH-Heissen /MH-Zentrum". The street continues as federal road B1. After 4 km of winding road you drive up a slope to a traffic light. Continue straight ahead, pass an ARAL gas station on your left, and after further 140 m the main road turns left (and downhill). Continue straight ahead into Bismarckstraße (parallel to the streetcar track - note the sign "Max-Planck-Institute" at this crossing). Follow the third road to the right (there is another sign "Max-Planck-Institute") for 300 m to reach the Institute, entrance Lembkestraße.

By train

Fast and comfortable train connections exist from all major German and European cities to either Essen (North and East) or Duisburg (South and West). From there local trains (S-Bahn, less than 10 min) take you to Mülheim Hauptbahnhof. Continue by taxi to the Institute, entrance Lembkestraße (5-10 min, ca. 5 Euro).

By air

From Düsseldorf Airport, it is most convenient to take a taxi to the Institute (22 km, 20-30 min, ca. 40 Euro). If the taxi driver is not familiar with Mülheim, instructions how to reach the institute by car are given above.

From Düsseldorf Airport there are also frequent direct train connections to Mülheim central station (Hauptbahnhof) which take 15 - 27 minutes depending on the type of train (IC, RE, S-Bahn, direction Dortmund).