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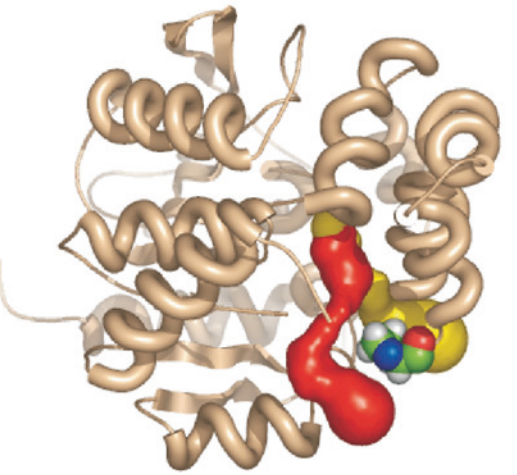
Protein Design and Evolution for Biocatalysis



Sant Feliu de Guixols
Spain
25-30 October 2008

Book of Abstracts

ESF-EMBO symposium

Protein Design and Evolution for Biocatalysis

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2008

Hotel Eden Roc
C/Port Salvi 57
17720 Sant Feliu de Guilxols
Costa Brava
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Programme

Day 1 - 25 October

Late afternoon	Registration at ESF-RC desk
19.00	Welcome drink
20.00	Supper

Day 2 - 26 October

08.45-09.00	Conference opening

Session 1: Fundamentals of enzymatic catalysis

Chairs: Paul Engel, University College Dublin, UK, Peter Neubauer, University of Oulu, FI

09.00-09.35	Stephen Benkovic Pennsylvania State University, US *A Perspective on Biological Catalysis*
09.35-09.55	Colin Jackson CSIRO, AU *Comparison Between Neutral Drift and Classical Evolution in the Development of Insecticide Resistance*
09.55-10.30	John Gerlt University of Illinois, US *Prediction of Function in the Enolase and RuBisCO Superfamilies*
10.30-11.00	Coffee break
11.00-11.35	Gregory Petsko Brandeis University, US *What Makes a Binding Site a Binding Site*
11.35-12.00	Florian Hollfelder University of Cambridge, UK *Multiple Catalytic Promiscuity*
12.00	Lunch
15.00-15.30	Coffee break

Session 2: Computer modelling in protein design

Chairs: Volker Heinrichs, Athenix, USA, Anju Chadha, IIT Madras, IN

15.30-16.05	Rebecca Wade EML Research, DE *Probing Enzyme Oligomerization and Regulation by Protein Mutation*
16.05-16.30	Emily Mundorff Codexis, USA *Development of the Codex Biocatalyst Panels*
16.30-17.00	Coffee break
17.00-17.35	Daniela Grabs-Roethlisberger University of Washington, US *Computational* de novo *Design of Protein Catalysts*

17.35-18.10	Sven Panke ETH Zurich, CH *Engineering Multi-Enzyme Systems*
19.00	Dinner
20.00-22.00	Poster session I *Authors with odd poster numbers*

Day 3 - October 27

Session 3: Computer modelling in protein design

Chairs: Miguel Gonzales, University of Barcelona, ES, Pierre Monsan, INSA University of Toulouse, F

09.00-09.35	Arieh Warshel University of Southern California, US *Hidden Principles of Enzyme Design*
09.35-09.55	Maria Suarez Ecole Polytechnique, F *Engineering of a Thioredoxin Protein with Additional Enzyme Function using Computational Design*
09.55-10.30	Federico Gago University of Alcala, ES *Computer Simulations of Enzyme Activity: Structural Snapshots of the Mechanism of Thioredoxin Reduction by* E. coli *Thioredoxin Reductase*
10.30-11.00	Coffee break
11.00-11.35	Adrian Mulholland University of Bristol, UK *Computational Enzymology as a Guide for Catalyst Design*
11.35-11.55	Sanja Tomic Rudjer Boskovic Institute, HR *Combined 3D QSAR and QM/MM Study of the* Burkholderia cepacia *Lipase Enantioselectivity*
11.55-12.30	Juergen Pleiss University Stuttgart, DE *Computational Enzyme Design: Structure, Dynamics, and Solvent Effects*
12.30	Lunch
15.00-15.30	Coffee break

Session 4: Bioinformatics in protein design

Chairs: Yan Feng, Jilin University, CN, Patrice Soumillion, Université Catholique de Louvain, BE

15.30-16.05	Janet Thornton European Bioinformatics Institute, UK *The Evolution of Enzyme Mechanisms and Functional Diversity*
16.05-16.25	Paul A. Bates Cancer Research, UK *Protein Engineering of the Cancer Drug: L-Asparaginase*

16.25-17.00	Brian Schoichet University of California - San Francisco, US *Forward and Reverse Chemical Information in Biology*
17.00-17.30	Coffee break
17.30-18.05	Janusz Bujnicki International Institute of Molecular and Cellular Biology, PL *Bioinformatics Analysis and Protein Engineering of Restriction Endonucleases*
18.05-18.40	Jan Kmunicek CESNET and Masaryk University, CZ *EGEE/EGI - the Infrastructure for* in silico *Experiments*
19.00	Dinner
20.30-21.30	Forward Look Plenary Discussion Chairs: Stephen Benkovic, Uwe Bornscheuer, Dick Janssen, Romas Kazlauskas, Manfred Reetz, Daniel Tawfik

Day 4 - October 28

Session 5: Directed evolution of biocatalysts

Chairs: Manfred Konrad, Max-Planck-Institute for Biophysical Chemistry, DE, Montarop Yamabhai, Suranaree University of Technology, TH

09.00-09.35	Dan Tawfik Weizmann Institute of Science, IL *The Makings of New Biocatalysts*
09.35-09.55	Ulrich Schwaneberg Jacobs University Bremen, UK *Steering Directed Protein Evolution: Strategies to Benchmark and to Manage Combinatorial Complexity of Mutant Libraries*
09.55-10.30	Manfred Reetz Max-Planck-Institute of Coal Research, DE *Methodology Development for Fast Directed Evolution*
10.30-11.00	Coffee break
11.00-11.35	Philipp Holliger MRC Cambridge, UK *Evolving Polymerases for the Synthesis and Replication of Nucleic Acids with Expanded Chemistry*
11.35-11.55	Aurelio Hidalgo Universidad Autónoma de Madrid, ES *Expanding the Short-chain Selectivity of* Pseudomonas fluorescens *Esterase I by Focused Directed Evolution and Rational Design*
11.55-12.30	Burckhard Seelig Harvard Medical School, US De novo *Enzyme Creation and Evolution Using mRNA Display*
12.30	Lunch
Afternoon	Half-day excursion to Girona
19.00	Dinner

20.00-22.00 Poster Session II
Authors with even poster numbers

Day 5 - October 29

Session 6: Directed evolution and engineering of biocatalysts

Chairs: Manfred Schneider, Bergische Universitaet, DE, Vytas Svedas, Lomonosov Moscow State University, RU

09.00-09.35 Romas Kazlauskas
University of Minnesota, US
Teaching Enzymes to Catalyze New Reactions

09.35-09.55 Amir Aharoni
Ben Gurion University, IL
Directed Evolution of Cytosolic Sulfotransferases for Enhanced Thermostability and Specificity

09.55-10.30 Karl-Erich Jaeger
Heinrich-Heine-University Duesseldorf, DE
Production and Design of Novel Biocatalysts

10.30-11.00 Coffee break

11.00-11.35 Karl Hult
Royal Institute of Technology, SE
Protein Engineering of Candida antarctica *Lipase B for New Substrate and Reaction Specificities*

11.35-11.55 Nobuhiko Tokuriki
Weizmann Institute of Science, IL
GroEL/ES Chaperones Promote Genetic Variation and Accelerate Enzyme Evolution

11.55-12.30 Stefan Lutz
Emory University, US
Engineering Enzymes by Circular Permutation

12.30 Lunch

15.00-15.30 Coffee Break

Session 7: Directed evolution and engineering for biocatalysis

Chairs: Magali Remaud-Simeon, University of Toulouse, F, Thomas John Smith, Sheffield Hallam University, UK

15.30-16.05 Uwe Bornscheuer
University Greifswald, DE
Rational Protein Design vs. *Directed Evolution: Examples to Improve Enantioselectivity*

16.05-16.25 Zbynek Prokop
Masaryk University, CZ
Choosy Enzyme Lego: Two Independent Enantioselective Elements Confined to a Single Active Site of Haloalkane Dehalogenase DbjA

16.25-17.00 Dick B. Janssen
Univeristy of Groningen
Engineered Enzymes for Enantioselective Epoxide Ring Opening

17.00-17.30	Coffee break
17.30-18.05	Nick Turner University of Manchester, UK *Directed Evolution of Enzymes for Applications in Organic Synthesis*
18.05-18.25	Marc Creus University of Neuchatel, CH *Artificial Metalloenzymes are Versatile Systems for Enantioselective (Bio)Catalysis*
20.00	Get-together & Conference Dinner

Day 6 - October 30

Breakfast & Departure

Abstracts of lectures

A Perspective on Biological Catalysis

Stephen J. Benkovic

Department of Chemistry, The Pennsylvania State University, 414 Wartik Laboratory, University Park, PA 16802, USA, sjb1@psu.edu

With dihydrofolate reductase as a paradigm, we have examined the question of the importance of conformational changes and their contributions to catalysis. A variety of collaborative approaches that include nuclear magnetic relaxation, pre-steady state kinetics, fluorescence resonance energy transfer, statistical correlation analysis, and molecular dynamic simulations have focused on the parent and mutant forms of the enzyme. The collective findings support the presence of a coupled network of residues within the protein fold that acts to generate a series of enzyme conformations along the reaction coordinate that optimize the reacting centers of the substrate and cofactor for the chemical transformation. The coupled network in turn may be modulated to affect the enzyme's activity leading to enzymes with allosteric properties.

Comparison between Neutral Drift and Classical Evolution in the Development of Insecticide Resistance

Colin J. Jackson,[1] Dan S. Tawfik[2]
and John G. Oakeshott[1]

1 CSIRO Entomology, ACT, Australia, colin.jackson@csiro.au
2 Department of Biological Chemistry, Weizmann Institute of Science, Rehovot, Israel

Neutral drifts are an emerging technique by which directed molecular evolution experiments can be performed. The E3 carboxylesterase from the sheep blowfly *Lucilia cuprina* is a rare natural example of both 'neutral drift' and classical enzyme evolution. The introduction of organophosphate (OP) pesticides in the early 20th century led to the development of enzymatic OP resistance via two separate pathways. Initially, the introduction of OPs resulted in the rapid enrichment of a pre-existing W251L mutation in E3 within pesticide resistant blowfly populations. This mutation confers improved OP hydrolase activity while maintaining wild-type esterase activity. After several years a second mutation arose (G137D) that rapidly superseded W251L in OP-treated regions. In contrast to the W251L mutation, G137D is detrimental to the native esterase activity. In this work we describe a directed evolution experiment in which the natural pathways to E3-mediated OP resistance are compared to those identified in an unconstrained laboratory setting starting from either the wild-type enzyme or a small but diverse neutral drift library. The distinct catalytic mechanisms that have evolved in E3 to confer phopshotriesterase activity, and their effects on the native esterase activity, are discussed in this context.

Prediction of Function in the Enolase and RuBisCO Superfamilies

John A. Gerlt

Departments of Biochemistry and Chemistry, University of Illinois, Urbana, IL 61801, j-gerlt@uiuc.edu

Determining the functions of proteins encoded by genomic sequences represents a major challenge in contemporary biology. We are developing an integrated sequence-structure-function strategy to facilitate functional assignment by predicting the substrate specificities of unknown proteins in the mechanistically diverse enolase and D-ribaulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO) superfamilies. The reactions catalyzed by members of both superfamilies are initiated by base-catalyzed abstraction of a proton from a carbon acid substrate to generate a Mg^{2+}-stabilized enolate intermediate (carboxylate anions in the enolase superfamily and ketose 1-phosphates in the RuBisCO superfamily).

We are using two approaches to assign functions to unknown members of both superfamilies: 1) experimental screening of libraries of potential substrates, and 2) computational prediction by in silico docking of libraries of potential substrates to both experimentally determined structures and homology models. In the RuBisCO superfamily, we discovered a novel isomerization reaction of 5-methylthio-D-ribulose 1-phosphate in an uncharacterized pathway for sulfur salvage. In the enolase superfamily, using library screening, we discovered several new acid sugar dehydratases, including L-fuconate dehydratase, D-tartrate dehydratase, and enzymes that catalyze the dehydration of both galactarate and L-talarate. Using the computational strategy, we successfully predicted several functions: N-succinyl Arg/Lys racemase, dipeptide epimerases with novel substrate specificities, and a divergent galactarate dehydratase. These latter successes establish that computational approaches provide a viable strategy to facilitate functional assignment of unknown enzymes discovered in genome projects. Supported by GM-71790.

What Makes a Binding Site a Binding Site

Melissa Landon, Dagmar Ringe
and **Gregory A. Petsko**

Departments of Biochemistry and Chemistry and Rosenstiel Basic Medical Sciences Research Center, Brandeis University, 415 South Street, Waltham, MA 02454-9110 USA, petsko@brandeis.edu

During the past 15 years, two techniques have been developed by one of us (DR) to map the complete binding surfaces of proteins whose trhee-dimensional structures have been determined. The first method, Multiple Solvent Crystal Structures or "solvent mapping", is the basis for all fragment-based drug discovery techniques that are in use in the pharmaceutical and biotech industries. It is an experimental approach that involves exposing protein crystals to relatively high concentrations of small molecules that represent organic functional groups. Determination of the protein crystal structures in the presence of these fragments enables their preferred sites of binding to be identified. Sites where multiple fragments cluster are the only places on the protein surface that seem capable of binding more complex drug molecules with high specificity and affinity. Recently, a computational version of this method has been developed by S. Vajda at Boston University; this implementation extends the method to homology models of target proteins, as well as actual crystalline macromolecules. The second approach to identifying binding sites, THEMATICS, involves the quantum-mechanical calculation of theoretical microscopic titration curves for every ionizable residue on the protein surface. Regions displaying residues with a particular kind of perturbed pKa values are likely binding sites for other molecules. The two different approaches give very good agreement, including finding many previously undetected sites, allowing us to define the general characteristics that make certain regions of the protein surface able to bind other molecules, while regions that seem superficially similar are never able to do so. We will present the general rules that have emerged form these studies, which emphasize the unique role of the first shell of bound water on the protein surface.

Multiple Catalytic Promiscuity

Bert van Loo, Luis Olguin, Ann Babtie, Stefanie Jonas
and **Florian Hollfelder**

*Department of Biochemistry, University of Cambridge,
Cambridge CB2 1GA, UK, fh111@cam.ac.uk*

'Promiscuous' enzymes possess activities in addition to their native ones. Promiscuous activities could be remnants from the evolutionary ancestor that has been gene duplicated and adapted to fullfill a new function. Alternatively the observation of promiscuity could indicate that an enzyme is 'pregnant' with another activity, i.e. has the potential to be evolved into a new catalyst. Thus the observation of promiscuity could define functional relationships in enzyme superfamilies, to serve a basis for drawing up phylogentic relationships for catalysis or active use in directed evolution.

We demonstrate this principle with observation of strong promiscuous activities with rate accelerations between 10^9 and 10^{15}. Promiscuity in one enzyme system is not limited to a single activity, but allows the same active site to catalyse up to six activities efficiently in addition to its native activity. We are starting to test whether the observation of catalytic promiscuity can serve as a shortcut to evolve new by interconversion within a promiscuous superfamily. Biological systems are compared to polymeric enzyme models that also show promiscuous features.

Probing Enzyme Oligomerization and Regulation by Protein Mutation

Rebecca C. Wade

Molecular and Cellular Modeling Group, EML Research, Schloss-Wolfsbrunnenweg 33, 69118 Heidelberg, Germany, rebecca.wade@eml-r.villa-bosch.de

Many enzymes function in an oligomeric state or as components of multi-protein complexes. Protein-protein interactions can affect the structure and dynamics of enzyme active sites and can play a role in enzyme activation and regulation. I will describe the application of protein-protein docking methodology[1] to predict the structure and stoichiometry of a multi-enzyme complex as well as the effects of mutations on the complex.[2] I will also discuss the use of computational approaches to identify hot-spot residues inside and outside a protein-protein interface that have a strong influence on complex formation. The identification of these residues may aid the design of ligands to interfere with protein-protein complex formation.

References:

[1] Motiejunas D., Gabdoulline R., Wang T., Feldman-Salit A., Johann T., Winn P.J., Wade R.C. (2008) Proteins 71: 1955-1969

[2] Feldman-Salit A., Wirtz M., Hell R., Wade R.C. (2008) J. Mol. Biol. in press

Development of the Codex Biocatalyst Panels

Emily Mundorff

Codexis, Inc., 200 Penobscot Dr, 94063 Redwood City, CA, USA, emily.mundorff@codexis.com

A Codex™ biocatalyst panel is a set of enzyme variants that are designed to accept a wide range of substrates, produce different stereoisomers, be chemically robust, and be manufactured at commercial scale. The plate variants are also designed to yield structure-function information that can be used to provide a jumpstart to evolution. To achieve these goals, a development strategy was conceived consisting of four steps: evolve a robust backbone, generate binding pocket diversity, screen on multiple diverse substrates, and select variants with broad phenotypic diversity. This strategy was then used to produce five Codex panels: ketoreductase, transaminase, ene reductase, acylase, and halohydrin dehalogenase. This strategy, as well as the development of two of these panels, the ketoreductase and ene reductase, will be presented.

Computational *de novo* Design of Protein Catalysts

Daniela Grabs-Röthlisberger

Department of Biochemistry, University of Washington, Seattle, WA, USA, roethlis@u.washington.edu

The design of novel enzymes capable of catalyzing any desired chemical reaction is a grand challenge for protein engineering and a critical test of our understanding of enzyme catalysis. Previous research in the Baker lab focused on protein structure prediction and design using computational methods. Development of RosettaMatch[1], which employs 6D hashing techniques to construct the catalytic machinery in a set of different protein scaffolds, facilitated introduction of enzymatic function onto the computational framework already employed in the lab. We recently described the computational design of several enzymes[2,3] for two reactions with non-natural substrates: the Kemp elimination – a model reaction for proton transfer from carbon – and a retro-aldol reaction – catalyzing the breaking of a carbon-carbon bond. Genes encoding the best scoring computational designs were then synthesized and expressed. Out of 59 experimentally characterized designs for the Kemp elimination, 8 showed catalytic activity. For this reaction both catalytic motifs were successfully employed with measured rate enhancements of up to 10^5 and multiple turnovers. Interestingly, the TIM barrel fold was the preferred scaffold for the Kemp elimination reaction as all active designs used this fold as protein backbone. For the retro-aldol reaction, 72 designs were experimentally characterized and 32 enzymes spanning a range of protein folds had detectable activity. Although four different catalytic motifs to catalyze the breaking of a carbon-carbon bond were employed, motifs utilizing an explicit water molecule to mediate proton shuffling were significantly more successful, with rate accelerations of up to 10^4 and multiple turnovers, than those involving charged sidechain networks. Mutation of the key catalytic residues significantly reduces or abolishes activity for both reactions. This result suggests that catalysis depends on the computationally designed active sites. High-resolution crystal structures of several of the enzymes for both reactions confirm that the designs have close to atomic accuracy.

References:

[1] Zanghellini A. *et al.* (2006) Prot. Sci. 15: 2785-2794
[2] Röthlisberger D. *et al.* (2008) Nature 453: 190-195
[3] Jiang L. *et al.* (2008) Science 319: 1387-1391

Engineering Multi-Enzyme Systems

Sven Panke

Bioprocess Laboratory, ETH Zurich, CAB H88, Universitaetsstrasse 6, 8092 Zurich, Switzerland, panke@ipe.mavt.ethz.ch

Synthetic Biology summarizes efforts directed at the synthesis of complex biological systems to obtain useful novel phenotypes based on the exploitation of well-characterized, orthogonal (=independent from the cellular context), and re-utilizable building blocks. It aims at recruiting design structures well established in classic engineering disciplines, such as a hierarchy of abstractions, system boundaries, standardized interfaces and protocols, and separation of manufacturing and design, for biotechnology. Synthetic Biology generally entertains the notion that successful design of a biological system from scratch - as opposed to the current practice of adapting a potentially poorly understood system over and over again - is the ultimate proof of fundamental understanding and concomitantly the most powerful way to advance biotechnology to the level required to deal with today's challenging problems in energy provision and the manufacturing of chemicals and pharmaceuticals.

We work towards exploiting the idea of orthogonality for metabolic engineering in order to make strain engineering more predictable. As we are mainly interested in typically phosphorylated intermediates of the central carbon metabolism, we use in vitro multi-enzyme systems imposed on cellular metabolism. Ultimately, we aim at providing insulated pathways the dynamics of which has been desensitized to cellular metabolic signals and adapted to manufacturing requirements. The latter goal requires quantitative, high-density measurements of comprehensive metabolite sets under dynamic conditions. We will present the results of our efforts towards comprehensive pathway insulation, high-throughput dynamic intermediate measurements, and pathway manipulation in the central carbon metabolism.

Hidden Principles of Enzyme Design

Arieh Warshel

Department of Chemistry, University of Southern California, University Park, 90089-1062 Los Angeles, CA, USA, warshel@usc.edu

Although the progress in enzyme engineering has been encouraging, the results of current designs are usually far less effective than the corresponding natural enzymes.

Moving forward requires us to answer two questions; a) what is missing in current designs and (b) how to obtain an improved design. Here we will focus on the first question and point out that the most important catalytic factor is the reduction of the reorganization energy of the polar environment in the active site. We will also demonstrate that this effect has not been considered in current design strategies.

We will start by explaining the preorganization concept and illustrating the problems in its analysis it the key case of KSI.[1] The effort needed for reliable calculations of transition state energies and reorganization energy will be discussed with clear test cases (e.g.[2]) and this will be used to asses the computer time needed for quantitative enzyme design. Finally a general strategy for enzyme design using a mutiscale modeling will be illustrated.

References:

[1] Warshel A., Sharma P.K., Chu Z.T., Aqvist J. (2007) Biochemistry 46: 1466
[2] Liu H., Warshel A. (2007) Biochemistry 46: 6011-6025

Engineering of a Thioredoxin Protein with Additional Enzyme Function using Computational Design

Maria Suarez,[1] Pablo Tortosa,[1] Maria M. Garcia-Mira,[2] David Rodríguez-Larrea,[2] Raquel Godoy-Ruiz,[2,3] Beatriz Ibarra-Molero,[2] Jose M. Sanchez-Ruiz[2] and Alfonso Jaramillo[1]

1 Laboratoire de Biochimie, CNRS-UMR 7654, Ecole Polytechnique, 91128 Palaiseau, France, maria.suarez@polytechnique.edu
2 Facultad de Ciencias, Departamento de Quimica Fisica, Universidad de Granada, 18071-Granada, Spain
3 Present address: Department of Chemistry and Biochemistry and Center for Biomolecular Structure and Organization, University of Maryland, College Park, Maryland 20742, USA

The scalable development of synthetic biology requires systematic methodologies to design molecular biological parts, especially multipurpose catalysts. We propose a new computational protein design methodology to engineer an enzyme, endowing it with a second, predefined, catalytic function. Moreover, computational design integrates the data from structural genomics into molecular models to engineer functional proteins by rewiring the complex network of atomic interactions. We have redesigned the high-plasticity positions surrounding the native active site to create a second active site catalyzing the new function. To avoid compromising protein folding and native activity, we have used a minimal perturbation approach based on the multi-objective combinatorial optimization of the de novo catalytic activity and the folding free energy. We experimentally validate our approach by introducing a promiscuous esterase activity in *E. coli* thioredoxin, by mutating positions near the native active site. Native oxidoreductase activity is not compromised, on the contrary, an insulin-reduction assay determines it to be 1.5-fold enhanced. This work shows how to use computational design to understand the evolvability of biological function by designing multipurpose proteins.

Computer Simulations of Enzyme Activity: Structural Snapshots of the Mechanism of Thioredoxin Reduction by *E. coli* Thioredoxin Reductase

Ana Negri, Esther Marco, Antonio Jiménez Ruiz
and **Federico Gago**

Grupo de Investigación Interdisciplinar, Departamentos de Farmacología y Bioquímica-Biología Molecular, Universidad de Alcalá, 28871 Alcalá de Henares, Madrid, Spain, federico.gago@uah.es

Thioredoxin reductase (TrxR; EC 1.6.4.5) from *Escherichia coli* is a homodimeric pyridine nucleotide-disulfide oxidoreductase that catalyzes the transfer of electrons from NADPH to thioredoxin (Trx). The electrons flow sequentially from (i) nicotinamide adenine dinucleotide phosphate (NADPH) to the flavin adenine dinucleotide (FAD) prosthetic group of oxidized TrxR, (ii) the reduced FAD to the redox-active disulfide composed of Cys135 and Cys138, and (iii) the resulting dithiol on TrxR to the disulfide on oxidized Trx. Reduced Trx, in turn, with active site residues Cys32 and Cys35, is a very efficient protein disulfide reductase that participates in many cellular processes including ribonucleotide reduction.

The crystal structure of *E. coli* TrxRox (PDB id 1TDE) revealed that each monomer consists of two globular domains connected by a double-stranded ß-sheet.[1] One domain contains the FAD-binding site whereas the other domain has the NADPH-binding site and the redox-active disulfide. The catalytic mechanism necessarily involves a conformational change that will bring the reduced Cys residues from the buried active site of TrxR to a pocket on the surface of the enzyme in which the disulfide of Trx will lodge in order to be reduced. In fact, the crystal structure of the covalently trapped complex between (C135S)TrxR and (C35S) Trx (PDB id 1F6M) revealed a "ball-and-socket" motion in which the NADPH-binding domain rotates by ~65º.[2] To study the switching between these two alternating conformations, we first analyzed the normal modes of vibration of TrxR and then docked Trxox using the automated docking program ClusPro.[3] The resulting TrxR-Trx complex was then simulated to gain atomic insight into the catalytic mechanism and the evolution of the substrate binding mode along the reaction pathway using a combination of targeted and unrestrained molecular dynamics simulations, as implemented in the program AMBER 8.0.[4] By following the reaction step by step we have been able to obtain a series of snapshots not easily amenable to experimental techniques that greatly facilitate understanding of some important mechanistic aspects of this fascinating enzyme. The importance of some Trx residues for binding to TrxR has been ascertained by site-directed mutagenesis (work done in J.M. Sánchez-Ruiz's laboratory in Granada, Spain).

References:

[1] Waksman G., Krishna T.S., Williams C.H. Jr, Kuriyan J. (1994) J. Mol. Biol. 236: 800-816
[2] Lennon B.W., Williams C.H. Jr, Ludwig M.L. (2000) Science 289: 1190-1194
[3] Comeau S.R., Gatchell D.W., Vajda S., Camacho C.J. (2004) Nucleic Acids Res. 32: W96-99
[4] Case D.A., Cheatham T.E.; Darden T., Gohlke H., Luo R., Merz K.M., Onufriev A., Simmerling C., Wang B., Woods R.J. (2005) J. Comput. Chem. 26: 1668

Computational Enzymology as a Guide for Catalyst Design

Adrian J. Mulholland

Centre for Computational Chemistry, School of Chemistry, University of Bristol, Bristol BS8 1TS, UK, adrian.mulholland@bris.ac.uk

Modelling of enzyme-catalysed reactions provides atomic-level knowledge of reaction mechanisms, and understanding of enzymes' catalytic properties, with the potential to contribute significantly in catalyst design. Combined quantum mechanics/molecular mechanics (QM/MM) methods are a good approach.[1] It is now possible to carry out QM/MM calculations on enzymes with high-level QM methods offering unprecedented accuracy.[2] QM/MM modelling allows mechanistic proposals to be evaluated, and can investigate the effects of mutations and identify catalytic interactions. Establishing enzyme chemical mechanisms is an essential first step, and is often challenging. An example is hen egg white lysozyme: QM/MM molecular dynamics simulations indicate that the reaction of the native enzyme with its natural substrate proceeds via a covalent intermediate, in contrast to the 'textbook' mechanism.[3] The role of protein dynamics in catalysis is another topic of lively debate. It may be important to mimic some aspects of enzyme dynamics and conformational behaviour in designed catalysts. Simulations are helping to identify and analyse functional protein motions. For example, modelling of reaction in citrate synthase suggests an unusual mechanism for coupling chemical and conformational changes.[4] Classical molecular dynamics simulations have identified large-scale cooperative motions relevant to the catalytic cycle in scavenger decapping enzyme.[5] Multi-level modelling of hydrolysis of the 'sleep inducer' oleamide by fatty acid amide hydrolase reveals complex conformational effects in catalysis6. The effects of quantum tunnelling can be analysed, e.g. in studies of aromatic amine dehydrogenase; it appears that protein dynamics do not 'drive' reaction in this case.[7] For some enzymes, such as chorismate mutase, QM/MM modelling identifies key positions for charged groups to achieve transition state stabilization, thus providing a template for catalyst design.[8]

References:

[1] Mulholland A.J. (2008) Biochem. Soc. Trans. 36: 22-26
[2] Claeyssens F. *et al.* (2006) Angew. Chem. Int. Ed. 45: 6856-6859
[3] Bowman A.L. *et al.*, Chem. Commun. in press
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[7] Masgrau L. *et al.* (2006) Science 312: 237–241
[8] Szefczyk B. *et al.* (2007) Int. J. Quantum Chem. 107: 2274-2285

Combined 3D QSAR and QM/MM Study of the *Burkholderia cepacia* Lipase Enantioselectivity

Sanja Tomic

Rudjer Boskovic Institute, Bijenicka 54, HR-10000 Zagreb, Croatia, sanja.tomic@irb.hr

We used two different approaches: 3D QSAR and QM/MM to investigate the high enantioselectivity of *Burkholderia cepacia* lipase (BCL) toward secondary alcohols.

First we identified possible binding modes for the series of the secondary alcohol esters in the BCL active site and derived the 3D QSAR model for predicting BCL enantioselectivity towards secondary alcohol. Afterwards, we investigate possibilities for chemical transformation of the secondary alcohol (*R,S*)-1-phenoxy-2-hydroxybutane[1] and its ester (E1) for which extremely high enatiomeric radio was measured (E>200).

Starting from the four covalent complexes with different orientation of the substrate (two determined by molecular modelling,[1] and two derived from the X-ray structure of the BCL-inhibitor complex[2,4]), we modelled the ester (*S* and *R* – E1) hydrolysis and the alcohol (*S* and *R* – 1) esterification using quantum chemical[3] (QM) and QM/MM4 methods. The calculations revealed that ester release is possible from all four covalent complexes. Alcohol release from the BCL-E1 complex in which the *S*-substrate is bound in the same manner as the *S*-inhibitor in the crystal structure however is not possible. The results proved that the productive binding modes of the secondary alcohol enantiomers were correctly predicted by molecular modelling and gave confirmation of the reliability of our 3D QSAR model.

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Computational Enzyme Design: Structure, Dynamics and Solvent Effects

Jürgen Pleiss

Institute of Technical Biochemistry, University of Stuttgart, Allmandring 31, 70569 Stuttgart, Juergen.Pleiss@itb.uni-stuttgart.de

Enzymes are useful catalysts with interesting biochemical properties such as high regio- and stereoselectivity, broad substrate spectrum, and high catalytic activity. Some enzymes such as lipases are also active in organic solvents. In principle, the biochemical properties of an enzyme can be predicted by modelling the binding of a substrate to the binding site of an enzyme on a molecular level. However, enzymes have a complex structure, and there is growing evidence of internal protein motions playing a crucial role in enzyme function. Moreover, the protein environment has an impact on structure and flexibility and, therefore, on enzyme function.

To establish a molecular model of selectivity and specificity, we have applied multiple molecular dynamics simulations of enzyme-substrate-solvent systems to study how the shape of the binding site, the geometry of the substrate, protein dynamics, and organic solvents mediate the biochemical properties of an enzyme. The effect of shape and flexibility to regioselectivity was studied for a human cytochrome P450 monooxygenase and for mutants of a bacterial monooxygenase.[1] The molecular basis of solvent effects was investigated by simulating explicit protein-solvent systems. Binding of water to a TEM-lactamase was shown to restrict the protein flexibility and to stabilize its structure,[2] while organic solvents reduced the flexibility of *C. antarctica* lipase B3 and induced large conformational transitions in lipases from *B. cepacia* and *R. miehei*. Detailed molecular models of enzymes were successfully applied to derive biochemical properties from first priniciples, to engineer reaction conditions, and to design enzymes.

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The Evolution of Enzyme Mechanisms and Functional Diversity

Janet M. Thornton,[1] Gemma L. Holliday,[1] Syed Asad Rahman[1] and John B. O. Mitchell[2]

1 EMBL-EBI, Wellcome Trust Genome Campus, Hinxton, CB10 1SD Cambridge, UK, thornton@ebi.ac.uk
2 UK Unilever Centre for Molecular Science Informatics, Department of Chemistry, University of Cambridge, Lensfield Road, CB2 1 EW Cambridge, UK

Enzyme activity is essential for almost all aspects of life. With completely sequenced genomes, the full complement of enzymes in an organism can be defined, and 3D structures have been determined for many enzyme families. Traditionally each enzyme has been studied individually, but as more enzymes are characterised it is now timely to revisit the molecular basis of catalysis, by comparing different enzymes and their mechanisms, and to consider how complex pathways and networks may have evolved.

To understand catalysis better we have also developed MACiE (Mechanism, Annotation and Classification in Enzymes), a database of the chemical mechanisms of enzymatic reactions. The MACiE dataset evolved from that published in the Catalytic Site Atlas (CSA) Porter et al. (2004) and each entry is selected so that it fulfils the following criteria. There must be a 3-dimensional crystal structure of the enzyme deposited in the Protein Databank (PDB); the mechanism is a relatively well understood mechanism; only one representative of each homologous protein family is included (H level of the CATH code - a hierarchical classification systems of protein domain structures - unless there is a homologue with a significantly different chemical mechanism. Details of each proposed enzyme mechanism are stored in a MySQL database. Research performed using MACiE to reveal which reaction steps are most common will be described.

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Protein Engineering of the Cancer Drug: L-Asparaginase

Marc N Offman,[1] Marcin Krol,[1] Naina Patel,[2]
Vaskar Saha[2] and **Paul A Bates**[1]

1 Biomolecular Modelling Laboratory, Cancer Research UK London Research Institute, Lincoln's Inn Fields Laboratories, London, UK, paul.bates@cancer.org.uk
2 Cancer Research UK Children's Cancer Group,Paterson Institute for Cancer Research, The University of Manchester, Manchester, UK

The bacterial enzyme, L-Asparaginase (L-Asn), is widely used in haematopoetic cancers such as acute lymphoblastic leukaemia (ALL). L-Asn, which is active as a tetramer, hydrolyses the amino-acid asparagine to aspartic acid and ammonia. Lymphoblasts only synthesise small amounts of asparagine; therefore, they are dependent on an exogenous supply. Administration of the drug L-Asn lowers asparagine blood levels and eventually leads to the apoptosis of leukemic lymphocytes.

L-Asn treatment is highly effective and has few side effects compared to standard chemotherapeutics. However, some patients do not respond to the treatment and become allergic to the drug. These side effects, are found primarily in high-risk patients and are caused by the degradation of L-Asn by two endopetidases. The degradation is responsible for rapid inactivation of L-Asn and an increased accessibility of antigenic epitopes.

Using a combination of structure and sequences analysis we identified the primary cleavage site. Experimental verification confirmed that a glycine mutation at this position result in a cleavage-resistant enzyme, albeit with a decreased activity. Further studies, employing genetic algorithms (GA) to search the sequence space at the primary cleavage site, combined with MD simulations, were applied to recover WT activity for a cleavage-resistant mutant. This resulted in the design of cleavage-resistant mutants with an increased activity of 23% compared to the WT.

Furthermore, we increased the stability of the L-Asn active tetramer, by the introduction of stabilising mutations at the monomer-monomer interface. Pre-selection of mutants was based on the amino-acid sampling frequencies calculated using our GA search engine. Mutants with the highest frequencies were subjected to free-energy of binding calculation; these free energies were used to select the final mutations. Preliminary experimental data confirmed our computational predictions and showed that a single point mutation at the interface can increase the activity by up to 200%. Ultimately, we intend to combine the best mutations in order to create a cleavage-resistant drug with a markedly increased activity and stability compared to the WT. This will enable therapy at a lower dosage, thus reducing the antigenic response to the drug.

Forward and Reverse Chemical Information in Biology

Brian K. Shoichet

Department of Pharmaceutical Chemistry, School of Pharmacy, University of California, San Francisco, 1700 4th Street, Byers Hall Room 508D, 94158-2330, San Francisco, CA, USA, shoichet@cgl.ucsf.edu

Bioinformatics Analysis and Protein Engineering of Restriction Endonucleases

Janusz M. Bujnicki[1,2]

1 Laboratory of Bioinformatics and Protein Engineering, International Institute of Molecular and Cell Biology in Warsaw, Poland, iamb@genesilico.pl
2 Institute of Molecular Biology and Biotechnology, Adam Mickiewicz University, Poznan, Poland

Restriction endonucleases (REases) are sequence-specific deoxyribonucleases used in vitro as reagents and tools in recombinant DNA protocols and medical diagnostics. They recognize short DNA sequences with extremely high specificity using multiple contacts per base pair. Typically, substrate recognition is also coupled to catalysis and mutation of residues involved in substrate recognition typically leads to inactivation of the cleavage activity. Therefore, rational engineering of REases has been largely limited to proteins with known structures, for which details of protein-DNA interactions could be elucidated. However, most of REases with known structures have proven refractory to engineering attempts. On the other hand, due to extreme sequence divergence it has been very difficult to obtain comparative models for other REases, with accuracy sufficient to predict protein-DNA contacts to be altered by mutagenesis. We have carried out comprehensive bioinformatics analysis of REases and selected several enzymes with substrate sequences containing unspecified bases (e.g. Bsp6I: GCNGC, NlaIV: GGNNCC), for which we could build structural models and predict residues or regions potentially involved in protein-DNA interactions. To this end, we have constructed a number of Bsp6I and NlaIV variants that discriminate different bases at previously 'unspecified' positions of the target DNA sequence.

EGEE/EGI - the Infrastructure for *in silico* Experiments

Jan Kmunicek

Institute of Computer Science, Masaryk University & CESNET, Botanicka 68a, 60200 Brno, Czech Republic, kmunicek@ics.muni.cz

Enabling Grids for E-sciencE (EGEE) EU project is building a large scale global compute and data storage infrastructure. The focus is to provide a shared collaborative environment where scientific teams with large compute or data intensive demands can realize their in silico experiments. EGEE spans the whole globe, with major sites located in all European countries, and many other in Americas and Asia Pacific region. With its 50000+ processors, 25PB storage located in 250+ sites it is the largest distributed infrastructure in the world. Support for large scale parametric studies, storage, retrieval and processing of huge datasets, coupled with the ability to keep information about the jobs run on the infrastructure makes EGEE an ideal environment to run extensive in silico experiments like the protein design. EGEE has been already used to search for new drugs against Asian Flu - in the WISDOM project - to help improve pediatry - in the Health-e-Child project - to model muscle cells and in many other activities where collaboration of different teams supported by shared computing resources is the key enabler for a successful scientific discovery, demonstrating high quality and value of the infrastructure. The EGEE is based on the gLite middleware, a set of components and services that keep care of user jobs and data regardless of the physical location. As EGEE serves also medical community, the security and privacy of data and jobs is also guaranteed.

EGEE currently sustains some 150 thousand jobs per day, keeping track of all of them. These “job trails” (provenance) can be used to supervise large scale simulations, semi-automatically resubmit jobs and together with appropriate user interfaces make the use of even this large infrastructure relatively easy job.

To make such an infrastructure sustainable and available to scientists in a longer term - the EGEE infrastructure has secured funding till April 2010 - European Grid Initiative Design Study (EGI_DS) EU project runs in parallel with EGEE to define a roadmap to the long term computing infrastructure. It will be based on national infrastructures, put together in a similar pattern like research computer networks are organized. Early adoption of EGEE infrastructure is thus secured to become a wise investment into future. With EGEE open to new scientific communities, the computational and data challenges of the protein design and evolution for biocatalysis are a perfect match for the EGEE infrastructure.

The Makings of New Biocatalysts

Dan S. Tawfik

Department of Biological Chemistry, Weizmann Institute of Science,
Rehovot 76100, Israel, tawfik@weizmann.ac.il

In spite the robustness and perfection of their mechanism of action, enzymes posses a remarkable ability to rapidly change and adopt new functions. I will describe how we generate new and efficient tailor-made enzymes starting from, either an existing enzyme with a weak, promiscuous function, or a computationally designed template. I will focus on newly developed methods to facilitate the directed evolution process, by using smaller libraries, and promoting enzyme evolvability using stabilizing ancestor/consensus mutations.

Steering Directed Protein Evolution: Strategies to Benchmark and to Manage Combinatorial Complexity of Mutant Libraries

Ulrich Schwaneberg

Jacobs University Bremen, Campus Ring 1, 28759 Bremen, Germany, u.schwaneberg@jacobs-university.de

Directed evolution of biocatalysts has become in academia and in chemical/pharmaceutical industries a widely accepted and broadly applied method. A directed protein evolution experiment comprises iterative steps of generating diverse mutant libraries and screening for improved protein variants. The quality of a mutant library is decisive for the success of a directed evolution experiment. Methods for generating randomized genes can be divided into three Categories (I-III)[1] depending on the method employed for generating genetic diversity: I) Enzyme based methods, II) Synthetic chemistry methods, and III) Whole cells methods. Enzyme based methods (I) comprise in vitro techniques for mutagenesis that use DNA polymerases or DNA modifying enzymes such as ligases, restriction enzymes and nucleases. Synthetic chemistry based methods (II) generate mutations via oligonucleotide-based synthesis, methods employing nucleotide analogues or DNA-modifying chemicals. In vivo random mutagenesis methods (III) use mutator strains or "mutator proteins" for generating mutations in host organisms. Most diversity generating methods use enzymatic or combined chemo-enzymatic approaches for mutagenesis. An ideal mutagenesis method would allow substituting every amino acid of a protein sequence by its 19 counterparts in a statistical manner. Current random mutagenesis methods fails in these attempts and suffer from three fundamental challenges: a) Bias of the polymerase or mutagenic agent that results in mutagenic "hot spots" and limits amino acid substitutions, b) Significant fraction of stop codons and destabilizing amino acid substitutions, and c) Lack of subsequent mutations due to methodological limitations. Combined with the redundant organization of the genetic code an average amino acid substitution per amino acid of 4-7 out of 19 can currently be achieved.[2]

A statistical tool named, Mutagenesis Assistant Program (MAP),[1] will be introduced to analyze the consequences of mutational bias on amino acid substitution patterns in proteins. MAP analysis reveals that conventional transition/transversion bias indicators fail on the protein level as benchmarks for mutagenic methods. Instead of transition/transversion bias indicators three indicators will be proposed that are based on the subset of amino acid substitutions generated by the employed mutagenic method. MAP has been implemented on an automatic server (http://gis-web.iu-bremen.de/MAP/) and provides upon DNA sequence input a statistical analysis for 19 random mutagenesis methods.

SeSaM, Sequence Saturation Mutagenesis, will further be introduced as conceptually novel and practically simple method that randomizes a target sequence at every single nucleotide position.[3,4,5] SeSaM has the potential to overcome the fundamental drawbacks of epPCR methods (see above a-c) and represents in its current stage a first step to solve the diversity challenge.

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Methodology Development for Fast Directed Evolution

Manfred T. Reetz

Max-Planck-Institute of Coal Research, Kaiser-Wilhelm-Platz 1,
45470 Mülheim am Ruhr, Germany, reetz@mpi-muelheim.mpg.de

Directed evolution of enzymes constitutes an attractive protein engineering method. Nevertheless, it remains most often time-consuming and laboratory-intensive. Therefore, current emphasis is on methodology development in the quest to make directed evolution more attractive for industry. Our contribution to this crucial endeavour is Iterative Saturation Mutagenesis (ISM). Two embodiments are possible, iterative CASTing for the control of substrate acceptance and enantioselectivity, and the B-FIT protocol for increasing the thermostability of proteins. New data and conceptual developments will be presented in the talk.

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Evolving Polymerases for the Synthesis and Replication of Nucleic Acids with Expanded Chemistry

Philipp Holliger

MRC Laboratory of Molecular Biology, Hills Road, CB2 2QH Cambridge, UK, ph1@mrc-lmb.cam.ac.uk

Nucleic acids not only transmit genetic information, but also can serve as versatile supra-molecular scaffolds. Our work is focused on expanding the substrate spectrum of polymerases with a view of enabling the synthesis and templated replication of nucleic acids with expanded chemistry. I'll be presenting recent progress on the generation of polymerases capable of substitution of canonical nucleotides with unnatural analogues, notably their fluorescent dye-labelled equivalents in PCR up to 1kb. The resulting "dyDNA" displays hundreds of heterocyclic dye molecules in a defined 3-dimensional arrangement. Consequently, dyDNA is not only brightly coloured and highly fluorescent but displays significantly altered physico-chemical properties such as organic phase partition and an increased apparent diameter as judged by atomic force microscopy. Some forms of dyDNA display extremely bright fluorescence enabling direct imaging of single dyDNA molecules in motion within a microfluidic flowcell. Polymerases capable of high-density incorporation of substituted nucleobases may aid the implementation of next-generation sequencing and arraying technologies, expand the chemical scope of nucleic acid enzymes and, ultimately, allow the encoded synthesis of novel polymers bringing evolution to the fields of nanotechnology and material science.

Expanding the Short-chain Selectivity of *Pseudomonas fluorescens* Esterase I by Focused Directed Evolution and Rational Design

Aurelio Hidalgo,[1,2] Esther Sánchez,[1] Anna Schließmann,[2] Rafael Molina,[3] Juan Hermoso,[3] José Berenguer[1] and Uwe T. Bornscheuer[2]

1 Centro de Biología Molecular "Severo Ochoa", UAM-CSIC, Madrid, Spain, ahidalgo@cbm.uam.es
2 Instituto de Química Física "Rocasolano", CSIC, Madrid, Spain
3 Institute of Biochemistry, Ernst-Moritz-Arndt-Universität, Greifswald, Germany

Protein engineering is currently performed either by rational design, focusing in most cases on only a few positions modified by site-directed mutagenesis, or by directed molecular evolution, in which the entire protein-encoding gene is subjected to random mutagenesis followed by screening or selection of desired phenotypes. A novel alternative is "focused directed evolution", in which only fragments of a protein are randomized while the overall scaffold of a protein remains unchanged. For this purpose, we developed a PCR technique (dubbed OSCARR) using long, spiked oligonucleotides, which allows randomizing of one or several cassettes in any given position of a gene. The high efficiency of this method was verified by creating focused mutant libraries of *Pseudomonas fluorescens* esterase I, screening for altered substrate selectivity, and validating against libraries created by epPCR. This led to the identification of a mutant within the OSCARR library with a 10-fold higher catalytic efficiency towards pnitrophenyl dodecanoate. This PFEI variant was modeled in order to explain the observed effects.

During the molecular modeling studies of the active site, the structural reasons for the short-chain substrate selectivity of PFEI were found to be in the restricted access to the active site, rather than in a small-sized substratebinding pocket. Consequently, four additional mutational hotspots were proposed, saturated and recombined, reaching improvements ranging from 10- to 150-fold in the hydrolysis of p-nitrophenyldodecanoate.

De novo Enzyme Creation and Evolution using mRNA Display

Burckhard Seelig[1,2]

1 Department of Molecular Biology and CCIB, Massachusetts General Hospital, Harvard Medical School, Boston, MA, USA, seelig@molbio.mgh.harvard.edu
2 Present address: Department of Biochemistry, Molecular Biology and Biophysics & BioTechnology Institute, University of Minnesota, Saint Paul, MN, USA

Enzymes isolated from nature are commonly modified and improved for specific applications by a wide range of methods such as screening, selection, directed evolution and rational design. However, the design and evolution of truly novel enzymes has relied upon extensive knowledge of the mechanism of the reaction.

Here, we show that genuinely new enzymatic activities can be created without the need for prior mechanistic information. We isolated several novel RNA ligases from a library of 4×10^{12} different proteins based on a zinc finger scaffold by using the mRNA-display technology with product formation as the sole selection criterion. The selected enzymes perform a template-dependent ligation of a 5'-triphosphate-activated RNA to a second RNA with a 3'-hydroxyl. There are no enzymes known in nature that catalyze this reaction. The novel enzymes exhibit rate enhancements of more than two million fold and show multiple turnover.[1] The ligases are currently improved further by in vitro evolution to achieve increased stability under denaturing conditions.

The approach described here, has the potential for the creation of a wide range of new enzymes and can also be used for the optimization and modification of known enzymes.

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Teaching Enzymes to Catalyze New Reactions

Romas Kazlauskas

BioTechnology Institute, University of Minnesota,
St. Paul, MN, USA, rjk@umn.edu

Evolution creates new catalysts from existing enzymes by mutagenesis to give them new abilities. One goal of biocatalysis is to recreate this evolution in the laboratory to produce unnatural enzymes that can solve current synthetic problems. There are three ways to create a new catalytic activity: 1) replace the active site metal ion with a new one, 2) extend the existing catalytic mechanism to analogs, often by changing substrate binding, and 3) create completely new catalytic steps. This lecture will discuss natural and engineered examples of each of these approaches.

Directed Evolution of Cytosolic Sulfotransferases for Enhanced Thermostability and Specificity

Amir Aharoni

Ben Gurion University in the Negev, Department of Life Science, Ben Gurion Blvd. 1, 84105 Beer-Sheva, Israel, aaharoni@bgu.ac.il

Cytosolic sulfotransferases (SULTs) are enzymes that detoxify an extremely broad range of xeno- and endobiotics by transfer of a sulfate to target acceptor compounds. These enzymes belong to a large family of detoxification enzymes, predominantly found in the mammalian liver, that exhibit broad specificity, relatively poor catalytic efficiency and, in many cases, inhibition at high substrate concentrations. The molecular basis for the broad specificity of SULTs has not been elucidated; for example, it is unclear how these enzymes utilize a diverse array of compounds and discriminate between foreign and physiologically relevant substrates. We have utilized directed evolution approaches to generate SULTs variants with enhanced thermostablity, catalytic activity and substrate specificity. Genetic diversity in the SULT1A1 and SULT1E1 isoforms was generated by DNA shuffling and random mutagenesis. The resulting libraries were screened following incubation at high temperatures for two SULTs substrates in order to identify and isolate clones with enhanced thermostability and substrate specificity. The novel SULTs mutants are currently thoroughly characterized using a variety of kinetic, biochemical and structural methodologies to shed new light on the molecular basis of the broad specificity of cytosolic SULTs family.

Production and Design of Novel Biocatalysts

Karl-Erich Jaeger, Thomas Drepper,
Sonja Tröscheland and Frank Rosenau

Institute of Molecular Enzyme Technology, Heinrich-Heine-University Düsseldorf,
Research Centre Jülich, D-52426 Jülich, Germany, karl-erich.jaeger@fz-juelich.de

White biotechnology depends on the availability of biocatalysts. Therefore, the demand is steadily growing to identify novel biocatalyst encoding genes and to efficiently produce biocatalyst proteins.[1] Three components are of prime importance for efficient biocatalyst production: the right gene, a suitable vector and an adequate expression host. A rapidly increasing number of available genome sequences exist as well as metagenomic gene libraries obtained from environmental DNA. However, the heterologous host bacterium *Escherichia coli* has an only restricted ability to express "foreign" genes encoding putative novel enzymes.[2] Here, we report on the construction of a series of shuttle vectors enabling the parallel screening of metagenomic libraries in *E. coli*, *Bacillus subtilis* and *Pseudomonas*. The newly constructed shuttle vectors possess a couple of useful features, such as (i) a comparatively small size, (ii) the option for GFP-monitoring of heterologous gene transcription, (iii) appropriate selection markers and (iv) strong xylose- and T7-inducible promoters.[3] We have further extended our expression tool box by construction of novel vectors and host strains allowing overexpression in the Gram-negative phototrophic bacterium *Rhodobacter capsulatus*.[4]

Apart from expressing single enzyme genes, the heterologous expression of natural product biosynthetic pathways is of increasing interest in biotechnology. Heterologous expression of gene clusters represents a major challenge. Bottlenecks include the construction and instability of appropriate expression vectors; inefficient transcription caused by poor recognition of foreign promoters, premature transcription termination and restriction to only few expression host strains. We have constructed a novel "in vivo auto cloning" (IVAC) and expression system consisting of two cassettes, named L- and R-IVAC.[5] The IVAC-expression system allows restriction-endonuclease independent cloning and stable integration of gene clusters in any Gram-negative host strain. In addition, both IVAC-cassettes contain viral T7 RNA-polymerase promoters to enable expression of complex gene clusters independent of the orientation of the respective single genes, their natural promoters and transcription terminators.

Correct folding is another necessary prerequisite to obtain enzymatically active enzymes. We discovered that the solubility of a given protein in *E. coli* which otherwise forms inclusion bodies can be significantly increased by coexpressing up to three different chaperones obtained from *Pseudomonas aeruginosa*.[6] The best respective combination of chaperones must be experimentally determined for each enzyme. Hence, we have constructed a chaperone toolbox which allows to identify the optimal chaperone combination for each individual enzyme.

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Protein Engineering of *Candida antarctica* Lipase B for New Substrate and Reaction Specificities

Karl Hult

Biocatalysis Group, Department of Biotechnology, Royal Institute of Technology, Roslagstullsbacken 21, 10691 Stockholm, Sweden, kalle@biotech.kth.se

Lipase B from *Candida antarctica* is a very versatile catalyst for many applications it is mainly know for its good enantioselectivity. The high enantioselectivity originates from a part of the substrate binding site which only easily accepts methyl or ethyl groups. Alteration of the amino acids forming this binding site changes the enantioselectivity. The size of this pocket can also be changed by water molecules that can bind to polar groups in the pocket. It is hypothesised that this small pocket is part of a water channel that supplies the active site with water for hydrolysis. Such a channel would allow water molecules to enter the active site without passing through the hydrophobic substrate phase. We hope to be able to discriminate hydrolysis over transacylation by blocking this passage for water. Therefore, we developed a screening method that can measure the ratio between acyl transfer to an alcohol and hydrolysis.

A restricted library of mutants involving three mutation points was expressed in *E. coli* and screened with the method shown in Fig. 1. Several mutants that preferred butanol over water were found. The mutants will be tested in transacylation reactions in water rich media as well as in polymerization reactions in which water restricts the length of polymers achieved.

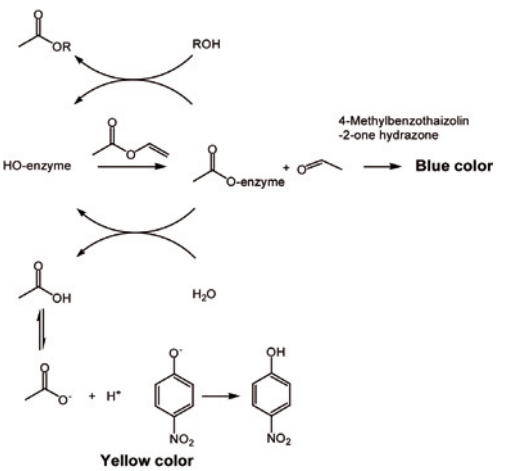

Figure 1. Screening method used to identify mutants with a high ratio of acyl transfer to alcohols over hydrolysis. Consumption of vinyl substrate and production of acid are followed colorimetrically. Acyl transfer to alcohol is calculated from the difference between substrate consumption and hydrolysis.

GroEL/ES Chaperones Promote Genetic Variation and Accelerate Enzyme Evolution

Tokuriki Nobuhiko

Department of Biological Science, Weizmann Institute of Science, 76100 Rehovot, Israel, nobuhiko.tokuriki@weizmann.ac.il

Theoretical and experimental studies suggest that seemingly neutral, or hidden, variation plays a key role in adaptation towards new environments, at the level of organisms and individual genes. However, most protein mutations, and mutations that alter function in particular, undermine stability, and deleterious. Thus, the destabilizing effects of mutations severely limit the acquisition of such neutral variation. Mechanisms that buffer these destabilizing effects are likely to facilitate the acquisition of hidden genetic diversity, and could thereby accelerate the rate of adaptation. Chaperones, or heat-shock proteins, are often implicated in buffering mutations.

We examined the buffering capacity of the bacterial GroEL/ES chaperones in conjunction with destabilizing and adaptive mutations. We performed mutational drifts (in vitro mutagenesis and selection for enzymatic function) of four different enzymes, GAPDH, PTE, hCAII and TIM in the presence of chaperone over-expression, and in its absence. In only few rounds of the neutral drift experiments, a significant fraction of GroEL/ES over-expression doubled the number of accumulating mutations and promoted the folding of enzyme variants carrying mutations in the protein core, and mutations with higher destabilizing effects (>3.5 kcal/mol ΔΔG values, on average, versus ~1 kcal/mol in the absence of GroEL/ES). The acquisition of a new enzymatic specificity occurred much faster under GroEL/ES over-expression, in terms of the number of improved variants (≥2-fold) and the improvements in specificity and activity (≥10-fold).

Our results provide direct evidence for the notion that GroEL rescue stability impaired mutants by increasing the fraction of soluble, functional enzyme, and thereby enable the accumulation of a larger number and variety of mutations, and therefore accelerate the rate of acquisition of new function. Finally, we propose this methodology as a new way of performing directed evolution in vitro.

Engineering Enzymes by Circular Permutation

Zheng Qian, Ying Yu and **Stefan Lutz**

Department of Chemistry, Emory University, Emerson 205, 1515 Dickey Drive, Atlanta, GA, USA, sal2@emory.edu

Circular permutation of enzymes offers a new approach for altering and improving catalytic performance; in particular in connection with the enantioselective synthesis and hydrolysis. Initially validated on the example of lipase B from *Candida antarctica* (CALB), circular permutation alters the structural integrity of the protein, changing its active site accessibility and flexibility, all factors affecting an enzyme's substrate recognition and turnover.[1,2]

Recent findings on the consequences of circular permutation on CALB structure, dynamics and function will be discussed and results on the techniques' broad application for protein engineering will be presented.

Acknowledgement: This study was financed in part by the US National Science Foundation (CRC-0404677 and CBET-0730312), as well as a grant from the Petroleum Research Fund of the American Chemical Society.

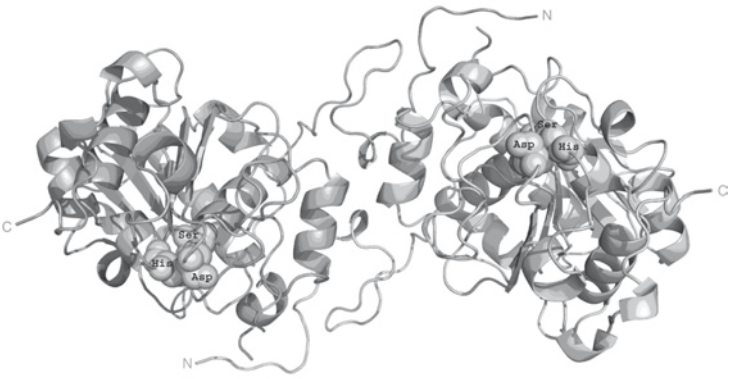

Figure 1. Crystal structure of cp283Δ7, a circular permutated CALB (PDB: 2R9D).[3]

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Rational Protein Design *vs.* Directed Evolution: Examples to Improve Enantioselectivity

Uwe T. Bornscheuer

Departement of Biotechnology & Enzyme Catalysis, Institute of Biochemistry, Greifswald University, Felix-Hausdorff-Str. 4, D-17487 Greifswald, Germany, uwe.bornscheuer@uni-greifswald.de

An impressive number of applications has been developed in the past decades for the use of enzymes in biotransformations with hydrolases as the most frequently used biocatalysts.[1]

Whereas initially, commercial enzyme preparations have been used 'straight from the bottle', the current trend is to tailor-design the biocatalyst using methods of protein engineering using rational design as well as directed evolution.

One of the most important but at the same time most challenging properties of biocatalysts is their stereoselectivity. Examples will be shown, in which high-throughput screening (HTS) systems for hydrolases[2] were successfully developed and applied to substantially improve the enantioselectivity of esterases for the synthesis of an important secondary alcohol serving as building block[3] as well as for tertiary alcohols. Here, first a sequence motif determining activity towards tertiary alcohols[4] was discovered followed by rational protein design[5] and focused directed evolution[6] to increase and invert selectivity and to broaden the substrate range.

More recently, we turned our interest on Baeyer-Villiger monooxygenase (BMVO). We could identify a new class of BVMO, which preferentially converts aliphatic ketones into the corresponding esters[7] and could demonstrate that for certain compounds, this reaction also proceeds with high enantioselectivity. Thus, hydroxyketones and 1,2-diols are now accessible using BVMOs in high yield and optical purity.[8] First examples for the improvement of enantioselectivity by directed evolution will be shown too.

Finally, catalytic promiscuity[9] will be introduced and first results[10] will be presented.

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Choosy Enzyme Lego: Two Independent Enantioselective Elements Confined to a Single Active Site of Haloalkane Dehalogenase DbjA

Zbynek Prokop,[1] Yukuri Sato,[2,5] Jan Brezovsky,[1] Jan Florian,[3] Tomas Mozga,[1] Radka Chaloupkova,[1] Tana Koudelakova,[1] Petr Jerabek,[1] Ryo Natsume,[5] Dick B. Janssen,[4] Yuji Nagata,[2] Toshiya Senda[5] and Jiri Damborsky[1]

1 Loschmidt Laboratories, Faculty of Science, Masaryk University, Kamenice 5/A4, 625 00 Brno, Czech Republic, zbynek@chemi.uni.cz
2 Department of Life Sciences, Graduate School of Life Sciences, Tohoku University, 2-1-1 Katahira, Sendai, 980-8577, Japan
3 Department of Chemistry, Loyola University Chicago, Chicago, Illinois 60626, USA
4 Department of Biochemistry, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands
5 Biomedicinal Information Research Center, National Institute of Advanced Industrial Science and Technology, 2-42 Aomi, Koto-ku, Tokyo 135-0064, Japan

The use of enzymes for preparation of optically pure building blocks – enantioselective biocatalysis - has emerged as an important tool in the 'green' industrial synthesis of pharmaceuticals and agrochemicals. Construction of enzymes possessing high enantioselectivities towards target molecules by protein engineering ultimately depends on our understanding of the structural basis of enantioselectivity. To broaden this understanding, we assayed three microbial haloalkane dehalogenases DhaA, LinB and DbjA for their enantioselective conversion of brominated ester and alkane substrates into chiral alcohols. All three enzymes possessed high enantioselectivity with brominated esters (E-value > 200), while DbjA additionally showed high enantioselectivity with β-substituted bromoalkanes (E-value ≤ 145).

Substrate mapping, mutagenesis, protein crystallography, thermodynamic analysis and theoretical modelling of the DbjA enzyme were used to explore enantiodiscrimination for each type of substrate. Brominated esters made three hydrogen bonds with a pair of halide-stabilising residues, while β-substituted bromoalkanes made two hydrogen bonds with the halide-stabilising residues as well as additional hydrophobic contact with the side of the active site pocket. Halide-stabilising residues are structurally conserved among different haloalkane dehalogenases making them highly enantioselective towards esters, while pockets and tunnels significantly differ among family members leading to different levels of enantiodiscrimination of β-substituted bromoalkanes. Consequently, enantiodiscrimination of β-substituted bromoalkanes is strongly affected by mutations introduced to the surface loop modulating the anatomy of the active site pocket, while the same mutations had no effect on the enantiodiscrimination of esters. Differences in the chemical nature of interactions involved in the formation of enzyme-substrate complexes, lead to distinct enthalphy and entropy contributions and differences in the role of (de)solvation in enantiodiscrimination.

Our interpretation of the observed enantioselectivity of DbjA represents a new paradigm, whereby a single enzyme active site is shown to possess two structurally uncoupled and chemically distinct bases for enantioselective discrimination of chiral molecules. This paradigm enables specific tailoring of enantioselectivity by mutagenesis, and opens new opportunities for engineering of catalysts for biocatalytic transformations.

Engineered Enzymes for Enantioselective Epoxide Ring Opening

Dick B. Janssen,[1] Marcus Schallmey,[1] Bert van Loo[1] and Maja Majerić Elenkov[2]

1 Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands, d.b.janssen@chem.rug.nl
2 Ruđer Bošković Institute, Bijenička c. 54, 10000 Zagreb, Croatia

Enzymatic epoxide ring opening is catalyzed by phylogenetically and mechanistically very different enzymes. Examples are 1) the classical epoxide hydrolases, which are related to the haloalkane dehalogenases and belong to the α/β-hydrolase superfamily of proteins; and 2) halohydrin dehalogenases, which belong to the short-chain reductase-dehydrogenase superfamily of proteins.

In classical epoxide hydrolases, ring-opening proceeds via covalent catalysis. Engineering efforts have been aimed at unraveling the role of catalytic residues and enhancing the enantioselectivity. Site-directed mutagenesis and directed evolution were used to enhance the enantioselectivity.[1] An interesting phenomenon in some mutants is that they developed sensitivity to substrate-conversion mediated inactivation, which appeared to be due to formation of isoaspartate from the nucleophilic aspartate during substrate turnover.

The halohydrin dehalogenases are involved in the bacterial degradation of haloalcohols and catalyze the conversion of vicinal haloalcohols to epoxides. Instead of a nicotinamide cofactor binding site which is common in SDR proteins, there is a halide-binding site. Based on the structure and kinetic properties of the halohydrin dehalogenase from *Agrobacterium radiobacter* (HheC), improved variants have been constructed with higher activity, enhanced enantioselectivity, and reduced sensitivity to proteolytic and oxidative inactivation.[2]

Both epoxide hydrolases and halohydrin dehalogenases catalyze nucleophilic epoxide ring opening. However, only the dehalogenases accept a variety of alternative nucleophiles, including cyanide, azide, formate, cyanate, nitrite and halides.[3] Various enantiopure products that are of interest for the preparation of fine chemicals can be formed this way.

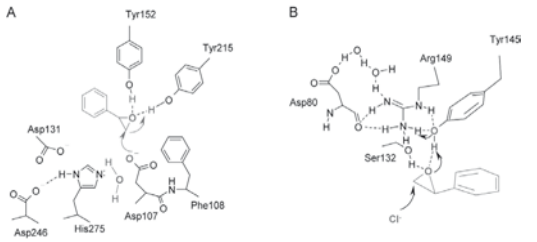

Figure 1. Epoxide ring-opening reaction catalyzed by *A. radiobacter* epoxide hydrolase (A) and halohydrin dehalogenase (B). The latter enzyme is highly promiscuous in nucleophile acceptance, allowing the production of a range of enantiopure β-substituted secondary or tertiary alcohols.

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Directed Evolution of Enzymes for Applications in Organic Synthesis

Nicholas J. Turner

School of Chemistry, Manchester Interdisciplinary Biocentre, University of Manchester, 131 Princess Street, M20 3DH Manchester, UK, nicholas.turner@manchester.ac.uk

The ability to change, and improve upon, the properties of a biocatalyst using directed evolution techniques has emerged as a powerful strategy in the past 10 years. By employing appropriately designed high-throughput screening methods, coupled with random mutagenesis to generate large libraries of enzyme variants, it is possible to identify biocatalysts with broader substrate tolerance, improved enantioselectivity and importantly enhanced properties (e.g. turnover, thermostability, solvent stability) when operating under process conditions. This lecture will review the state-of-the-art in this area, including examples from our own laboratory, in which a range of different enzymes (e.g. amine oxidases,[1-5] alcohol oxidases,[6-7] racemases, ammonia lyases, P450 monooxygenases[8]) have been subjected to directed evolution to change one or more properties. A key focus has been the development of deracemisation reactions, or their equivalent, in which chiral amines, alcohols and amino acids are obtained in high yield and enantiomeric excess.

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Artificial Metalloenzymes are Versatile Systems for Enantioselective (Bio)Catalysis

Marc Creus and Thomas R. Ward

Department of Chemistry, Faculty of Sciences, University of Neuchatel, Emile Argand 11, CP 158, CH-2009 Neuchatel, Switzerland, marc.creus@unine.ch

We have exploited supramolecular anchoring strategies to ensure the localization of a catalytically active metallic moiety within a chiral protein scaffold, using avidins as protein hosts. The enantioselective reactions that we have implemented thus far include: hydrogenation, transfer hydrogenation, allylic alkylation and sulfoxidation. Recently, we have also expanded the technology to include kinetic resolution via phosphoester hydrolysis. Artificial metalloenzymes, at the interface between chemistry and biology, offer insights into the mechanisms of (bio)catalysis and enzyme evolution.

Abstracts of posters

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P 2 ***Andre Isabelle*** Insights into molecular motions of *Burkholderia cepacia* lipase inferred from mixed molecular modelling and robotic based path planning approaches

P 3 ***Awadelkarim Mohamed*** Directed evolution of an existing phenylalanine dehydrogenase, L307V, for enantiopure synthesis of phenylalanine analogues substituted at the 2- and 3-position of the aromatic ring

P 4 ***Bar Hagit*** The application of ancestral resurrection for protein engineering – the AChE model case

P 5 ***Binay Baris*** Construction of 246Gly and 240Ala mutant for changing the substrate specificity of the *Bacillus sterothermophillus* lactate dehydrogenase (bs LDH)

P 6 ***Brezovsky Jan*** Modulation of enantioselectivity in haloalkane dehalogenase DbjA by engineering of a surface loop

P 7 ***Brissos Vânia*** Development of directed evolution assays to improve the redox potential of CotA-laccase

P 8 ***Butz Maren*** Tunable protein production system allows directed evolution of highly active enzymes

P 9 ***Chadha Anju*** The versatile biocatalyst for deracemisation: Mechanism and scope of *Candida parapsilosis* ATCC 7330

P 10 ***Chaloupkova Radka*** Thermodynamic analysis of enantioselectivity of haloalkane dehalogenase DbjA and its variants DbjAΔ and DbjAΔ+H139A towards brominated esters and β-substituted bromoalkanes

P 11 ***Chen Sihong*** Directed evolution of phenylalanine dehydrogenase from *Bacillus sphaericus* towards activity with an un-saturated non-natural amino acid

P 12 ***Chilov Ghermes*** Application of molecular docking methods for modeling enzyme specificity

P 13 ***Chovancova Eva*** HotSpot Wizard: Web server for identification of hot spots for mutagenesis experiments

P 14 ***Copp Janine*** Identification and improvement of novel bacterial nitroreductases for cancer gene therapy

P 15 ***Daroch Maurycy*** Isolation and preliminary characterisation of *S. aeruginosa* peroxidase with potential industrial relevance

P 16 ***Desmet Tom*** Directed evolution of disaccharide phosphorylases

P 17 ***Dyguda-Kazimierowicz Edyta*** Phosphotriesterase-catalyzed hydrolysis of sarin: QM/MM simulation of the reaction pathway

P 18 ***Fairchild Steven*** Engineering proteins for enhanced activity

P 19 ***Feng Yan*** The novel substrate lineages by key motif directed module recombination

P 20 ***Ferrario Valerio*** Steered molecular dynamics and GRID for simulation of lipase activation

P 21 ***Fuhrmann Markus*** Slonomics – A novel technology for the generation of highly designed gene libraries

P 22 ***Gaidukov Leonid*** Directed evolution and mechanistic studies of serum paraoxonases

P 23 ***Gatti-Lafranconi Pietro*** (Un)Predictable effects of distant mutations on protein activity and stability

P 24 ***Giger Lars*** Illuminating the mechanism of a PLP-dependant aldolase using an *in vivo* selection system

P 25 ***Goedl Christiane*** Molecular mechanisms of glucosyl transfer in retaining disaccharide phosphorylases

P 26 ***González Miguel*** QM/MM investigation of the reactions of the hepatitis C virus NS3 protease with its main natural substrates: Reaction mechanism and molecular dynamics simulations

P 27 ***Gumulya Yosephine*** Iterative saturation mutagenesis based on B-factors to increase thermostability of *Aspergillus niger* epoxide hydrolase

P 28 ***Heinrichs Volker*** Improvement of protein thermostability, solubility and enzymatic activity by directed evolution

P 29 ***Hoebartner Claudia*** Engineering a selective small-molecule substrate binding site into a deoxyribozyme

P 30 ***Iranzo Casanova Olga*** Designing *de novo* superoxide dismutases

P 31 ***Jesenska Andrea*** Dynamics of water molecules at the tunnel mouths of haloalkane dehalogenases studied by time resolved fluorescence spectroscopy and computer simulations

P 32 ***Jochens Helge*** Converting an esterase into an epoxide hydrolase

P 33 ***Jones Aubrey*** Directed evolution of two key cellulases for the conversion of biomass to ethanol

P 34 ***Joosten Henk-Jan*** 3DM: A tool for optimal use of superfamily data for enzyme engineering

P 35 ***Khersonsky Olga*** Directed evolution of *in silico* designed enzymes

P 36 ***Klvana Martin*** Pathways and mechanisms for product release in engineered haloalkane dehalogenase DhaA explored using classical and random acceleration molecular dynamics simulations

P 37 ***Knapic Lorena*** 3D-QSAR model for the quantitative prediction of enantioselectivity of lipase B from *Candida antarctica*

P 38 ***Konrad Manfred*** Cellular delivery of catalytically improved nucleoside kinases to enhance metabolic activation of prodrugs and improve anticancer and antiviral therapy

P 39 ***Kotik Michael*** Modification of activity and enantioselectivity of an epoxide hydrolase by saturation mutagenesis and novel epoxide hydrolases from environmental DNA

P 40 ***Koudelakova Tana*** Analysis of substrate specificities of wild-type haloalkane dehalogenases and their mutants by multivariate statistics

P 41 ***Krol Marcin*** Protein engineering of the cancer drug: L-Asparaginase

P 42 ***Kufka Julia*** Mechanistic studies and modification of prenylating enzymes

P 43 ***Lafaquière Vincent*** A structured-controlled lipase enantioselectivity investigated by a path planning approach

P 44 ***Leitner Christian*** Improving the activity of pyranose 2-oxidase via site directed mutagenesis of the active site loop

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P 47 ***Martins Ligia*** Development of directed evolution assays to improve the redox potential of CotA-laccase

P 48 ***Mazura Pavel*** Rational design and directed evolution of the maize beta-glucosidase Zm.p60.1

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P 50 ***Muellegger Johannes*** ZymeCAD, a versatile and comprehensive molecular simulation platform: Application in the study of antigen-antibody complexes

P 51 ***Munawar Nayla*** *In vitro* reactivation of inclusion bodies and homologous expression of recombinant glutamate dehydrogenases from *Halobacterium salinarum*

P 52 ***Narayanan Rajesh*** Kinetic studies of phenylalanine dehydrogenase from *Bacillus sphaericus* and derived mutants with a variety of substrates

P 53 ***Nerinckx Wim*** An upside-down substrate flip within the active site of evolutionary related beta and alpha glycopyranoside hydrolases explains their mechanistic relationship

P 54 ***Neubauer Antje*** EnBase: High density cell cultivation for high throughput screening

P 55 ***Neubauer Peter*** TIM-based biocatalyst platform for chiral synthesis of chemical compounds

P 56 ***Ochoa-Leyva Adrian*** Protein design for new activities through catalytic loop exchange in the (βα)8-fold

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P 58 ***Pawlak Sebastian*** Rational design of restriction endonuclease specificity through computational analysis and subunit rearangement

P 59 ***Rannes Julie*** Directed evolution of new galactose oxidase variants for glycan labelling

P 60 ***Remaud-Simeon Magali*** Tailoring alpha-transglucosidase specificity for programmed chemo-enzymatic synthesis of complex bacterial oligosaccharides

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P 62 ***Sawayama Andrew*** A Panel of cytochrome P450 BM3 variants to produce drug metabolites and diversify lead compounds

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P 64 ***Schneider Sarah*** Changing the substrate scope of transaldolase B *E. coli* towards non-phosphorylated compounds

P 65 ***Schneider Manfred*** Carba analogues of PAF: Enzyme assisted syntheses and biological activities

P 66 ***Schreier Bettina*** Engineering the enolase active site pocket: Is the second magnesium ion essential for enolase activity?

P 67 ***Schuermann Martin*** Directed evolution and specific engineering of industrial biocatalysts for enantioselective C-C-bond formation

P 68 ***Shubina Tatyana*** New cage compounds as potential influenza A inhibitors: Synthesis and docking/QSAR study

P 69 ***Skowronek Krzysztof*** Engineering of the NlaIV restriction endonuclease sequence specificity

P 70 ***Smith Thomas John*** Mutagenesis and directed evolution of key bacterial monooxygenases toward improved biocatalysis

P 71 ***Snajdrova Radka*** Towards biocatalysts for organosilane biotransformations

P 72 ***Soni Pankaj*** B factor iterative test (B-FIT) for enhancing enzyme stability in organic solvents

P 73 ***Soumillion Patrice*** Engineering a beta-lactamase allosterically regulated by kanamycin

P 74 ***Soumillion Patrice*** Everything is there but it is not a beta-lactamase

P 75 ***Spadiut Oliver*** Improving pyranose 2-oxidase from *Trametes multicolor* for biocatalytic applications by genetic engineering

P 76 ***Spelberg Jeffrey Lutje*** Biocatalyst panels: Using tunable biocatalysts to accelerate process development

P 77 ***Strerath Michael*** Evolution of screening assays during the extensive phenotypic remodeling of an unspecific human serine protease towards higher activity, high selectivity and blood serum inhibitor insensitivity

P 78 ***Stroganov Oleg*** Molecular modeling of substrates recognition by penicillin acylase

P 79 ***Suarez Maria*** Construction of a library of orthogonal transcription factors with their corresponding promoters based on design of synthetic zinc-finger proteins

P 80 ***Suplatov Dimitry*** Bioinformatic analysis of penicillin acylase family reveals key residues essential for enzyme stability

P 81 ***Svedas Vytas*** Molecular modeling of nucleophile binding and reactivity in penicillin acylase-catalyzed acyl transfer

P 82 ***Syrén Per-Olof*** Rational redesign of *Candida antarctica* lipase B for kinetic resolution of bulky sec-alcohols

P 83 ***Takwa Mohamad*** Rational design of *Candida antarctica* lipase B for the ring opening polymerization of lactides

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P 85 ***Tkaczuk Karolina*** Comprehensive evolutionary analysis, comparison of active sites and re-classification of the SPOUT superfamily of methyltransferases

P 86 ***Valentini Maria*** Characterization of site-specific interactions of the MTGase enzyme with G-CSF: Why is Q134 PEGylated and Q131 is not?

P 87 ***van Loo Bert*** A Most promiscuous enzyme: Six reactions catalyzed by one active site

P 88 ***Villiers Benoit*** Substrate promiscuity and directed evolution of a nonribosomal peptide synthetase adenylation domain (TycA)

P 89 ***Thomas Veena*** Stability and activity constrain evolution of an antibiotic resistance enzyme

P 90 ***Weignerova Lenka*** The alpha-galactosidase type A gene from *Aspergillus niger* encodes a fully functional a-N-acetylgalactosaminidase

P 91 ***Whitehead Sarah*** Novel tandem reactions and the directed evolution of a promiscuous bifunctional enzyme

P 92 ***Yamabhai Montarop*** Improvement of endochitinase activity by directed evolution

P 93 ***Yelboga Emrah*** Site saturation mutagenesis of crucial residues in NAD/NADP specificity of formate dehydrogenase from *Candida methylica*

P 94 ***Yuan Bo*** Selective oxidation of primary and secondary alcohols using variants of galactose oxidase

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P 96 ***Zhang Guirong*** Directed evolution of a hyperthermophilic esterase from the archaeon *Aeropyrum pernix* K1 to improve the hydrolysis efficiency towards plant cell wall

For authors presenting posters, panels measuring **140** cm high x **100** cm wide will be available at the conference site.

Poster sessions will be held on Sunday and Tuesday: **20.00 to 22.00**.

Authors with **odd** poster numbers should be available at their posters during the session on **Sunday** (Poster Session I), keeping their posters on display until Tuesday morning.

Authors with **even** numbers will present on **Tuesday** (Poster Session II) and keep their posters on display until the end of the conference.

P 1

The Gradual Evolution of New Enzymes via Multi-step Loop Replacements

Livnat J. Afriat and Dan S. Tawfik

Department of Biological Chemistry, Faculty of Biochemistry, Weizmann Institute of Science, Herzl street 1, 76100 Rehovot, Israel, livnat.afriat@weizmann.ac.il

Our study focuses on the role of active-site loops in enzymes in mediating the acquisition of new functions. Almost all laboratory evolution experiments involved point mutations. In Nature, however, replacements in the form of insertions and deletions of loop segments ("loop swaps") seem to underline the divergence of numerous enzyme families and superfamilies. PTE is a phosphotriesterase from P. diminuta with a TIM-barrel fold. This enzyme is thought to have evolved towards the degradation of paraoxon, a synthetic insecticide introduced in the 20th century. We have identified a new group of lactonases, which we named PLLs (PTE-like lactonases). Although these lactonases evolved >100 million years ago, they possess a latent, promiscuous phosphotriesterase activity. We hypothesized that, once phosphotriester insecticides were introduced, PTE evolved from an existing PLL by virtue of this latent promiscuous activities.[1] The ancient lactonases (PLLs) and the modern PTE share key sequence and active site features but differ primarily in the length and sequence of two active site loops. We are using the sequence and structure comparisons of these enzymes, and directed evolution, to gradually convert PTE into its putative lactonase progenitor. So far we have engineered several variants that show a shift in substrate specificity towards the lactonase activity; while the phosphotriesterase activity decreases up to 10,000 folds the lactonase activity increases up to 50 folds. The most profound differences follow changes in surface loops. Albeit, the promising changes in substrate specificity also led to reduced expression and stability. We are currently attempting to combine the individual loop changes together with point mutations, in order to isolate the putative lactonase precursor of PTE.

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P 2

Insights into Molecular Motions of *Burkholderia cepacia* Lipase Inferred from Mixed Molecular Modelling and Robotic based Path Planning Approaches

S. Barbe, V. Lafaquière, D. Guieysse, J. Cortés, P. Monsan, T. Siméon, M. Remaud-Siméon and **Isabelle André**

Laboratoire d' Ingénierie des Systèmes Biologiques et des Procédés, UMR CNRS 5504, UMR INRA 792, F-31400 Toulouse, France, isabelle.andre@insa-toulouse.fr
LAAS-CNRS, 7 av. Du Colonel-Roche, 31077 Toulouse, France

The use of enzymes as biocatalysts is of a great industrial interest for the preparation of chiral building blocks, especially by kinetic resolution of racemic mixtures. In this field, lipases are among the most employed enzymes. Widely distributed in nature, these enzymes catalyze the hydrolysis and the synthesis of a wide range of soluble and insoluble organic compounds, making them potential catalysts for a wide variety of applications in chemical, pharmaceutical and food industries. The interest of using lipases lies in their enantioselectivity which has been shown to be modulated by reaction conditions, such as the temperature or the solvent employed. Nonetheless, structural determinants and molecular motions controlling lipase activity and selectivity are not yet fully understood.

In this context, we studied the role of the movement of a sub-domain covering the active site of *Burkholderia cepacia* lipase (BCL) on its activation as well as the influence of the substrate accessibility to BCL active site on the enzyme enantioselectivity.[1] Atomic-resolution of such slow-timescale molecular motions is out of reach for currently available experimental techniques. Therefore, computational methods are needed to complement experimentation. For this purpose, a mixed approach combining both molecular modelling techniques and motion planning algorithms originally used in robotics[2,3] was developed by the LAAS-CNRS and applied to investigate BCL molecular motions. Compared to classical molecular modelling techniques, this novel approach allowed a performance gain of several orders of magnitude (hours vs weeks) to compute continuous large amplitude protein motions in solvent environment and accessibility pathways of substrates from the protein surface to a buried protein active site.

Computational simulations were used to drive directed mutagenesis experiments to enhance lipase activity and enantioselectivity further. At a more general level, this fast technique could be used as a pre-filtering procedure to select a catalyst or accelerate the engineering of a given catalyst for a given racemate resolution.

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P 3

Directed Evolution of an Existing Phenylalanine Dehydrogenase, L307V, for Enantiopure Synthesis of Phenylalanine Analogues Substituted at the 2- and 3-position of the Aromatic Ring

Mohamed A. Awadelkarim and Paul C. Engel

School of Biomolecular and Biomedical Science, Conway Institute, University College Dublin, UCD, Belfield, Dublin 4, Ireland, mohamed.awadelkarim@ucd.ie

Recent advances in molecular biology techniques coupled to high-throughput screening methods have paved the way for developing novel biocatalysts with desired specificity and stability under different reaction conditions. As described in the literature, biocatalysis has now become a powerful industrial tool which can be applied in the synthesis of fine chemicals, pharmaceuticals and agrochemicals, owing to the unique features of biocatalysts which make them superior to traditional non-biological catalysts.[1,2] Engineered phenylalanine dehydrogenases (PheDHs) from *Bacillus sphaericus*[3] proved to be useful as biocatalysts for the synthesis of enantiopure nonnatural amino acids.[4,5] In the present work, an existing mutant, L307V PheDH,[3] was predicted to have a deep binding pocket for access of substrates with large aromatic side-chains, and might prove useful for the synthesis of phenylalanine analogues substituted at the 2- and 3-position of the aromatic ring. Kinetic characterisation of the enzyme should distinguish the effects of the mutation at position 307 on the affinity and on the maximum catalytic rate of the enzyme for substrates substituted at the 2- or 3-positions of the aromatic ring. Furthermore, attempts to enhance the activity of the existing mutant by random mutagenesis and screening assisted by an automated colony picker will be reported.

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P 4

The Application of Ancestral Rresurrection for Protein Engineering – the AChE Model Case

Bar Hagit

Department of Biological Chemistry, Faculty of Life Science, Weizmann Institute of Science, Ullmann building, room 206, Herzl street 1, 76100 Rehovot, Israel, hagit.bar@weizmann.ac.il

The unique biochemical properties and physiological significance of acetylcholinesterse make it an interesting target for protein engineering. However, past attempts to functionally express mammalian acetylcholinesterase's, and in particular human acetylcholinesterase, in adequate amounts in *E. coli*, failed. We believe that engineering a more thermostable human acetylcholinesterase can facilitate its soluble expression in *E. coli*, and open the road for further engineering this important enzyme.

Despite many successes, the routinely used methods of engineering highly stable proteins lack generality, and produce mixed and sometimes undesirable results such as changes in activity or specificity.

The primary aim of this work is to assess and develop methods based on ancestral resurrection for the engineering of stabilized proteins for bacterial expression and directed evolution. To this end, we have reconstructed a phylogenetic tree of all acetylcholinesterase's and inferred the most probable ancestor of all mammalian acetylcholinesterase's. Subsequently, we have constructed a library that contains the most probable ancestor sequence, and sequences that relate to alternative ancestral states at ambiguously predicted positions (<95% prediction). An initial screen of the library resulted in the isolation of variants which were 5 up to 10 times more active than wild type, contemporary human acetylcholinesterase in *E. coli* crude lysate. Sequencing revealed that the most active variants have high sequence similarity to the most common ancestor with 3 amino acids divergence between the two. However, preliminary thermostability assays imply that these "near ancestor" variants do not exhibit higher stability than the wild type human acetylcholinesterase. This might exclude the ancestor from being the most stable or soluble variant of a protein family and implies the existence of a "local maxima" that is situated in the sequence space between the contemporary genes (e.g. hAChE) and their inferred ancestors. In the search for the "local maxima", we have constructed a combinatorial library of all possible AChE sequences between the mammalian ancestor and human contemporary AChE. The best variants were isolated and sequenced, and the mutations which confer the highest improvement were identified. Next, we will use comparative thermodynamic and expression measurements of the consensus, ancestor, and the best expressed AChE variants (the "local maxima"), which combined with sequence analysis will allow us to infer general conclusions regarding the minimal changes required for thermostabilizing and solubly expressing our chosen enzyme.

P 5

Construction of 246Gly and 240Ala Mutant for Changing the Substrate Specificity of the *Bacillus stearothermophillus* Lactate Dehydrogenase (bs LDH)

Baris Binay

Molecular Biology and Genetics, Science and Letter Faculty, Istanbul Technical University, Maslak, 34469 Istanbul, Turkey, binay@itu.edu.tr

Lactate dehydrogenase from thermophilic organism *Bacillus stearothermophilus* (bs LDH) has a crucial role in producing chirally pure hydroxyl compounds. Alpha hydroxy acids are used to produce various pharmaceuticals such as semisynthetic penicillin using S-α-hydroxyacids. Having very limited substrate specificity[1] is one disadvantage of this enzyme. L-lactate dehydrogenase exhibits only barely detectable activity levels towards another 2-hydroxyacid, L-mandelate. We expect to change the activity of bsLDH to react with L-mandelate by using protein engineering techniques. Here, we have successfully introduced substantial mandelate dehydrogenase activity into the enzyme with Insight II molecular modeling program. Modelling studies suggest that two mutations namely; Thr246Gly and Ile240Ala will allow the enzyme to utilize L-mandelate as a substrate2 at the end of the energy minimization.

Mutations have been introduced into bsLDH gene in 6xHis-tag pQE-2 expression vector by using Invitrogen Gene Tailor™ site-directed mutagenesis system. Both wild type and mutant LDHs have been produced. Following the purification of both LDHs, kinetic studies of the enzyme will be carried out and the results will be compared with the wild type bsLDH protein.

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P 6

Modulation of Enantioselectivity in Haloalkane Dehalogenase DbjA by Engineering of a Surface Loop

Jan Brezovsky,[1] Zbynek Prokop,[1] Yukuri Sato,[2,4] Jan Florian,[3] Tomas Mozga,[1] Radka Chaloupkova,[1] Tana Koudelakova,[1] Petr Jerabek,[1] Ryo Natsume,[4] Yuji Nagata,[2] Toshiya Senda,[4] and Jiri Damborsky[1]

1 Loschmidt Laboratories, Faculty of Science, Masaryk University, Kamenice 5/A4, 625 00 Brno, Czech Republic, briza@chemi.muni.cz
2 Department of Life Sciences, Graduate School of Life Sciences, Tohoku University, 2-1-1 Katahira, Sendai, 980-8577, Japan
3 Department of Chemistry, Loyola University Chicago, Chicago, Illinois 60626, USA
4 Biomedicinal Information Research Center, National Institute of Advanced Industrial Science and Technology, 2-42 Aomi, Koto-ku, Tokyo 135-0064, Japan

Haloalkane dehalogenase DbjA is highly enantioselective towards structurally simple molecule 2-bromopentane (E = 145), while two other family members, DhaA and LinB, show low enantioselectivity with this substrate (E = 7 and E = 16, respectively). Structural analysis revealed that DbjA contains unique surface loop in its specificity-determining domain. Deletion of this loop lead to the mutant enzyme DbjAΔ with significantly lowered enantioselectivity with 2-bromopentane (E = 58). Enantioselectivity could be re-introduced by additional single-point mutation DbjAΔ+H139A (E = 120).

We have used computer modeling to understand how the surface loop of DbjA modulates enantiodiscrimination of linear brominated alkanes. The property of the binding sites of haloalkane dehalogenases DbjA, DhaA and LinB was quantitatively compared using comparative binding energy (COMBINE) analysis. This analysis revealed that DbjA has clearly separated stabilizing and destabilizing van der Waals interactions on each side of the active site due to its distinctively wide active site pocket. Both (*R)*- and (*S)*-enantiomer binds along the same wall of the active site, which provides stabilization for the alkyl chain by hydrophobic interactions. The free energies of binding for individual binding modes were calculated using linear response analysis (LRA) and revealed that (*R*)-enantiomer shows lower binding energy compared to (*S)*-enantiomer. The preference calculated for (*R*)-enantiomer from differential binding is significantly increased when population of near attack configurations (NACs) are considered. Calculated data correctly reproduce the observed changes in enantioselectivity in DbjAΔ and DbjAΔ+H139A. Deletion of the loop results in rotation of His139, narrowing down the active site pocket. Interactions with His139 displaces (*R*)-enantiomer from its reactive position and leads to significant drop in enantioselectivity of DbjAΔ mutant (NACs = 19.7 %, E_{calc} = 71 in DbjA *versus* NACs = 6.4 %, E_{calc} = 49 in DbjAΔ). Additional mutation of His139Ala reconstitutes width of the active site pocket, reactivity of (*R*)-enantiomer and enantioselectivity of DbjAΔ+H139A to its original level (NACs = 24.1 %, E_{calc} = 79).

These results indicate that wide and solvated active site is important for enantioselective discrimination of structurally simple molecule 2-bromopentane by DbjA, and explain why DhaA and LinB with the narrow access tunnel discriminate linear brominated alkanes poorly. Our study further demonstrates that enantioselectivity of enzymes can be modulated by the surface loop engineering which may have important implications for construction of new enantioselective biocatalysts.

P 7

Development of Directed Evolution Assays to Improve the Redox Potential of CotA-laccase

Vânia Brissos,[1] Luciana Pereira,[1] Florentina-Daniela Munteanu,[3] Artur Cavaco-Paulo[2] and Lígia O. Martins[1]

1 Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Av. Da República, 2780-157 Oeiras, Portugal, vbrissos@itqb.unl.pt
2 Departamento de Engenharia Têxtil, Universidade do Minho, 4800-058 Guimarães, Portugal
3 Present address: University Aurel Vlaicu of Arad, Department of Chemical and Biological Sciences, Elena Dragoi St, 2, 310330 Arad, Romania

Laccases, that belongs to the multicopper oxidase family of enzymes, are useful biocatalysts for many and diverse biotechnological applications. Substrate oxidation by laccases involves the Marcus "outer-sphere" mechanism in which the redox potential (E0) difference between the substrate and the T1 Cu site of these enzymes (together with the reorganization energy and the electronic coupling) determines the electron transfer and thus, the enzymatic oxidation rate. Laccases with higher E0 have increased effectiveness and versatility for substrates avoiding the need of redox mediators in many applications. Our studies on the structure-function relationships revealed the limitation of rational approaches to engineer the E0 showing that changes of amino acid residues in direct contact to the metal centre significantly affect the properties of T1 Cu sites of laccases and the enzyme overall reactivity and stability.[1,2] Directed evolution has merged in the past few years as a powerful alternative to rational approaches for engineering biocatalysts. The key to the directed evolution process is the establishment of an efficient expression system and screening system that accommodates the predicted diversity generated by the mutagenesis techniques. In this study the expression levels of cotA were compared in five different *Escherichia coli* host strains, growing in 96 well microtiter plates, under different cultivation conditions. The lower coefficient of variance (15%) was achieved in high-throughput screening assays for standard laccase substrates using crude cell lysates of BL21 and KRX host strains growing under microaerobic conditions. By plotting the decolourisation values of 12 different anthraquinonic and azo dyes by CotA-laccase and the E0 of dyes (measured by cyclic voltammetry) a direct correlation could be observed in accordance with results in the literature. Sensitive and reproducible high-throughput decolourisation screening assays were developed for the identification of high redox variants from evolution libraries. The enzymatic assays developed were tested for the screening of a library (around 2000 clones) from Domain 3 of CotA laccase created by error-prone PCR. The mutagenesis and screening strategies selected allowed for the identification of a variant enzyme with a 2 fold higher activity than the parental strain, therefore suitable for parenting the next generation of random mutagenesis.

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P 8

Tunable Protein Production System Allows Directed Evolution of Highly Active Enzymes

Maren Butz

Department of Chemistry and Applied Biosciences, ETH Zurich, Wolfgang-Paulistrasse 10, 8093 Zurich, Switzerland, butz@org.chem.ethz.ch

Directed evolution of highly active enzymes consists of two major steps: first, the creation of genetic diversity in the target gene in the form of gene-libraries, and second, an efficient selection system for the desired catalytic activity.

For *in vivo* selections, the growth phenotype of a mutant depends on the total enzyme activity present within the cell, which is determined by the specific activity of the catalyst and also its concentration. A minimal enzyme activity is necessary for cell survival. Upon increase of the total enzyme activity, the cells divide faster, and the selection system can be used to identify catalysts with improved specific activities. However, there is an upper limit of total enzyme activity; above this limit only maximal cell growth is observed. If the starting total enzyme activity in a given selection system is close to or even higher than this limit, the selection system can not be used for further improvement of the catalyst. This particular issue was encountered in our attempts to improve a chrismate mutase mutant which showed wild-type like growth phenotype. To overcome this problem we created an adapted selection system that allows the catalyst to be limited to very low intracellular concentrations. The first modified selection system relied on a very efficient C-terminal degradation tag and an inducible promoter.[1] The second improved selection system involves an N-terminal degradation tag and the same promoter. The second selection system has the advantages of allowing randomization of the whole gene and reducing false positives by a factor of ten.

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P 9

The Versatile Biocatalyst for Deracemisation: Mechanism and Scope of *Candida parapsilosis* ATCC 7330

Anju Chadha, T. Vaijayanthi, D. Titu, Tarjan, Kumar, Nidhi Agarrwal, Kamal Joshi, Vaibhav and P. Mahajabeen

Laboratory of Bioorganic Chemistry, Department of Biotechnology, IITM, 600036 Chennai, India, anjuc@iitm.ac.in

Candida parapsilosis ATCC 7330 is a versatile biocatalyst for the deracemisation of alpha- and beta-hydroxy esters as shown by us earlier. The predominant enantiomer that is formed is 'S' via a stereoinversion mechanism. The most likely enzymes involved in this whole cell biotransfomration are the 'R' specific oxidase and an 'S' specific reductase. Expanding the scope of this biocatalyst to accept more and varied substrates some new and exciting experimental results have lead us to understand the substrate preferences of this biocatalyst using computational tools. The presentation will give an update on our experimental and computational work with this biocatalyst.

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P 10

Thermodynamic Analysis of Enantioselectivity of Haloalkane Dehalogenase DbjA and its Variants DbjAΔ and DbjAΔ+H139A towards Brominated Esters and β-substituted Bromoalkanes

Radka Chaloupkova,[1] Zbynek Prokop,[1] Tomas Mozga,[1] Yukuri Sato,[2,3] Tana Koudelakova,[1] Yuji Nagata,[2] Toshiya Senda[3] and Jiri Damborsky[1]

1 Loschmidt Laboratories, Faculty of Science, Masaryk University, Kamenice 5/A4, 625 00 Brno, Czech Republic, radka@chemi.muni.cz
2 Department of Life Sciences, Graduate School of Life Sciences, Tohoku University, 2-1-1 Katahira, Sendai, 980-8577, Japan
3 Biomedicinal Information Research Center, National Institute of Advanced Industrial Science and Technology, 2-42 Aomi, Koto-ku, Tokyo 135-0064, Japan

Newly isolated haloalkane dehalogenase DbjA from *Bradyrhizobium japonicum* USDA110 shows high enantioselectivity towards brominated esters and β-substituted bromoalkanes. Structural analysis and comparison of amino acid sequence of DbjA with sequences of other family members detected the presence of inserted fragment unique to DbjA which is located on the protein surface. Construction and characterization of variant DbjAΔ carrying deletion of the extra amino acid sequence showed that the fragment is involved in enantioselectivity of DbjA with β-substituted bromoalkanes, but not with the brominated esters.

Detailed comparison of crystal structures of wild type DbjA and deletion mutant DbjAΔ enzymes revealed that floppy amino acid residue H139 could be involved in modulation of chiral recognition of β-substituted bromoalkanes, resulting in construction of variant DbjAΔ+H139A. Its kinetic characterisation revealed modulation in enantioselectivity towards β-substituted bromoalkanes and no changes in enantioselectivity with brominated esters.

To elucidate origin of enantiomeric discrimination of brominated esters and β-substituted bromoalkanes, thermodynamic analysis was conducted with selected substrates and three enzyme variants DbjA, DbjAΔ and DbjAΔ+H139A. Measurement of temperature dependence of enzyme enantioselectivity provides information on differential activation enthalpy and entropy and their changes in different enzyme variants. Differential activation enthalpy was found to be major determinant of chiral recognition for brominated esters by all three enzyme variants. Enzyme enantioselectivity towards brominated esters could be explained by different binding interactions of individual enantiomers with the residues of the active site in Michaelis complex and/or the transition state of dehalogenation reaction. On the other hand, entropy was found to play equally important role as enthalpy for enantiomeric discrimination of 2-bromopentane by DbjA and DbjAΔ+H139A. Interestingly, DbjAΔ showed reversed dependence of enantioselectivity on temperature for 2-bromopentane when entropy dominated over enthalpy contribution towards enzyme enantioselectivity. Importance of entropy for kinetic resolution of β-substituted bromoalkanes by all studied enzymes can be related to different flexibility of both enantiomers in the enzyme active site and different solvation/desolvation of enzyme active site upon binding.

The results from thermodynamic analysis let us conclude that: (i) DbjA possess two chemically distinct bases of enantioselectivity towards brominated esters and β-substituted bromoalkanes and (ii) protein variants with very different thermodynamic contributions towards enantioselectivity can be constructed by protein engineering

P 11

Directed Evolution of Phenylalanine Dehydrogenase from *Bacillus sphaericus* towards Activity with an Un-saturated Non-natural Amino Acid

Sihong Chen

School of Biomolecular and Biomedical Science, Conway Institute, University College Dublin, Belfield 4, Dublin, Ireland, sihong.chen@ucd.ie

Phenylalanine dehydrogenases (EC 1.4.1.20) (PheDH) catalyse reversible deamination of phenylalanine and some of its analogues to the corresponding keto acids using NAD+ as coenzyme. By directed evolution, we are altering the substrate specificity of PheDH to make biocatalysts for synthesis of other amino acids including the unsaturated non-natural amino acid L-propargylglycine, which is of great interest for organic synthesis.

Two rounds of medium-rate error-prone PCR were conducted on the PheDH gene from *Bacillus sphaericus*. After screening around 2000 colonies, several mutants showed improved activity with the target substrate. After overexpression and purification, one mutant, 1B6, showed catalytic efficiency (Vmax/Km) increased 5.9-fold with DLpropargylglycine and 2.3-fold with L-Phe. Further random mutation of 1B6 resulted in 5B10 and 5C2, showing catalytic efficiency increased 6.3 and 5.9-fold with DL-propargylglycine and 1.7and 2.5-fold with L-Phe compared to wild type. Homology modelling using the solved structure of PheDH from *Rhodococcus* sp. M4 suggested that these mutations are far away from the active site.

In search of more efficient mutants, one round of high-rate epPCR was performed. Around 3000 colonies were screened with the aid of a colony picker and automation workstation. After overexpression of "positives" and purification, one mutant, H14A12, showed 3.4-fold improvement of catalytic efficiency towards DL-propargylglycine and 2025-fold decrease towards L-Phe compared to wild type. Therefore this mutant showed 7500-fold improvement of selectivity, but is not a good catalyst. Another mutant, H7H10 displayed 7.1-fold and 6.9-fold increase of catalytic efficiency towards the target substrate and natural substrate compared to wild type PheDH. Homology modelling suggested that G124C in H14A12 and E313G in H7H10 are near the active site.

Saturation mutagenesis targeting the two positions, G124 and E313 has now been performed and about 4000 colonies were screened. Several mutants displayed improved activity with the target substrate. Further kinetic assay of these purified mutant enzymes with the target and natural substrates will be reported. Better PheDH mutants with higher activity towards target substrate and better selectivity between the target and natural substrate are expected.

P 12

Application of Molecular Docking Methods for Modeling Enzyme Specificity

Ghermes Chilov

MolTech, Ltd., Leninskie gory, 1/75A, 119992 Moscow, Russian Federation, ghermes@moltech.ru

Molecular docking is a well-established technique for assessment of protein-ligand interactions and is routinely used in drug discovery process to find potent binders for particular target protein and to optimize ligand's binding affinity. However, accuracy of currently available docking methodologies is still far from desired, and this obstacle diminishes the competence of docking as a single source of proof, and forces to maintain large proportion of laborious experimental work and other computational approaches (say QSAR). Obviously, applications of docking in biocatalytic tasks are also hampered since modeling enzyme specificity and especially rational enzyme design needs even more accuracy. However, novel molecular docking software Lead Finder by MolTech, Ltd has demonstrated significant improvement of accuracy: docking success rate (correct prediction of enzyme-substrate structure) comprised 85-96% on all publicly available test-sets used in validation of other commercial docking software (such as Glide, Gold, FlexX, LigandFit, MolDock, Surflex), and prediction of ligand binding affinity with 1.5 kcal/mol accuracy (validated on the set of 330 diverse protein-ligand complexes). Lead Finder software has been recently applied in the field of biocatalysis - to study enzyme specificity and rational enzyme design. A set of about 70 penicillin acylase (PA) substrates and inhibitors representing several different chemotypes (aliphatic and aromatic alcohols, substituted phenylacetic acid derivatives, phenylacetylated amino acids, β-lactam antibiotics) with known binding constants (ranging from sub-millimolar to micromolar) was used to validate ability of Lead Finder to predict ligand's binding affinity. It appeared that RMSD of predicted vs experimental binding energies was about 0.9 kcal/mol, with overall qualitatively correct ranging of binding affinity within ligands of certain chemotype and among different chemotypes. Ability of Lead Finder to dock ligands and calculate accurately their binding energies was used in prediction of PA enantioselectivity (E) in the hydrolysis of phenylacetyl amino acids. Good quantitative correlation of calculated and experimentally measured E-values was observed. Further improvement of predicted Evalues was achieved by molecular dynamical (MD) modeling of enzyme complexes with enantiomeric substrates and averaging binding energy and statistics of near attack conformations over 10 nanosecond MD trajectory. Further applications of molecular docking and molecular dynamics methods concern rational enzyme design. Docking technology may be used for generating complexes of mutant enzyme(s) with substrate and assessment of corresponding binding energy and reactivity. With fast and precise tool of molecular docking millions of mutant enzyme forms can be screened in silico providing valuable mutations for immediate experimental check or incorporation into directed evolution scheme. Selected mutants can be subjected to more exhaustive molecular modeling studies (including molecular dynamics and QMMM calculations) to gain more sound conclusions on the rational enzyme design.

P 13

HotSpot Wizard: Web server for Identification of Hot Spots for Mutagenesis Experiments

Antonin Pavelka, **Eva Chovancova** and Jiri Damborsky

Loschmidt Laboratories, Faculty of Science, Masaryk University, Kamenice 5/A4, 62500 Brno, Czech Republic, akllupe@chemi.muni.cz

During the evolution, certain regions in an enzyme structure change more rapidly than others, reflecting different evolutionary constraints. Amino acids essential for maintaining structural or functional properties of particular enzyme tend to be conserved over evolution, whereas other sites are permissible towards substitutions. Highly conserved sites are generally not suitable targets for mutagenesis experiments due to their key role for structural stability or functionality. Mutagenesis targeting the variable positions can be regarded as more safe. Mutagenesis of the highly variable sites, which at the same time participate on substrate binding or transition state stabilization, may possibly lead to the modulation of catalytic activity and substrate specificity.

HotSpot Wizard is a web server for automatic identification of such hot spots through integration of structural and evolutionary information obtained from selected bioinformatics databases and computational tools. The server requires a structure of the query protein in PDB format as the only input. In the first step, Catalytic Site Atlas and Uniprot databases are used to determine residues indispensable for protein function. HotSpot Wizard then searches for potentially important residues by CASTp, identifying the active site cavity, and by CAVER, calculating tunnels connecting buried cavities with the outside solvent. Finally, evolutionary conservation of individual amino acid positions in the query protein is estimated by CONSURF from multiple sequence alignment of closely related proteins. In the output, HotSpot Wizard lists residues ordered by predicted mutability together with information on conservation level and potential function. Results are mapped on the protein structure and can be visualized directly in the web browser using JMOL applet or downloaded as the input for PyMOL visualization software.

HotSpot Wizard offers an easy way to perform several structural and evolutionary analyses at once with minimal demands on users, making our server possibly useful for experimentalists with no prior knowledge of computer modelling. HotSpot Wizard is freely accessible at http://loschmidt.chemi.muni.cz/hotspotwizard/.

P 14

Identification and Improvement of Novel Bacterial Nitroreductases for Cancer Gene Therapy

Janine N. Copp,[1] G.A. Prosser,[1] S.P. Syddall,[2] A.V. Patterson[2] and D.F. Ackerley[1]

1 School of Biological Sciences, Victoria University of Wellington, PO Box 600, 6140 Wellington, New Zealand, janine.copp@vuw.ac.nz
2 Auckland Cancer Society Research Centre, University of Auckland, New Zealand

NfsB, a nitroreductase (NTR) from *Escherichia coli*, is capable of converting nitroaromatic prodrugs, such as 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB1954), to their cytotoxic derivatives. The combination of NfsB and CB1954 is one of several enzyme/prodrug strategies that have generated considerable interest as potential cancer therapies. Indeed, expression of NfsB can increase the sensitivity of tumour cells to CB1954 by greater than 1000-fold. However, suboptimal kinetics hinder NfsB activation of CB1954 at clinically achievable doses.

The discovery and evolution of novel NTRs with enhanced prodrug activation kinetics is therefore of considerable benefit to cancer gene therapy. To achieve this we have developed a screen for prodrug activation based on the 'SOS-chromotest', which employs an *E. coli* strain which expresses a reporter gene in response to DNA damage. We have used this simple and rapid technique to successfully identify novel enzymes that can activate CB1954 and other prodrugs. Several candidates display enhanced activity when compared to NfsB.

Initial screens of mutant error prone libraries generated from our novel NTR candidates have highlighted the potential of this approach to evolve superior NTRs for use in cancer gene therapy.

P 15

Isolation and Preliminary Characterisation of *S. aeruginosa* Peroxidase with Potential Industrial Relevance

Daroch Maurycy

University of Liverpool, Faculty of Sciences, Department of Biological Sciences, Biosciences Building, Crown St., L69 7ZB Liverpool Merseyside, UK, M.Daroch@liv.ac.uk

Peroxidases are a group of versatile enzymes widely distributed in nature, possessing significant biotechnological potential for oxidations and selective epoxidations of synthetic intermediates. Some peroxidases possessing oxidative halogenating activities were found in lignin-degrading basidiomycetes and a link between ligninolytic cycle and biological halogenation has been hypothesised. Initial screening of basidiomycete strains known to be good producers of organohalogens was performed using assays based on o-dianisidine and monochlorodimedone, in a search for novel peroxidases. The screening results showed that the 'magic' mushroom, *Stropharia aeruginosa*,secretes extracellular peroxidase(s) exhibiting some halogenating activity when grown on high nitrogen content medium. The extracellular peroxidases were purified to a high degree using a combination of ultrafiltration and chromatographic procedures, yielding two major isoforms differing in their degree of N-glycosylation. Both peroxidase isoforms showed identical peptide sequences when analysed by tandem mass spectrometry. Degenarate oligonucleotides designed from those peptide sequences are being used to screen a cDNA library of *S. aeruginosa* in pursuit of the peroxidase gene. If this approach proves successful, the peroxidase will be heterologously expressed in a eukaryotic system and tested in biotransformation assays for synthetic organic chemistry applications and subjected to engineering if some of the features required modification.

P 16

Directed Evolution of Disaccharide Phosphorylases

Desmet Tom, De Groeve Manu and Soetaert Wim

Department of Biochemical and Microbial Technology, Faculty of Bioscience Engineering, Ghent University, Coupure Links 653, 9000 Gent, Belgium, t.desmet@ugent.be

The in vivo role of DSPs is to catalyse the degradation of disaccharides with the help of anorganic phosphate. This results in the production of a glycosyl phosphate, which can be metabolised through glycolysis without further activation by a kinase. DSPs can, however, also be used for the in vitro synthesis of glycosidic bonds because phosphorolysis is a reversible reaction. The main advantage of these enzymes is that they only require glycosyl phosphates as donor instead of the expensive nucleotide-activated donor sugars that are needed by glycosyltransferases. Unfortunately, the vast majority of DSPs are limited to the transfer of a glucosyl residue and are not able to use e.g. galactose-1-phosphate as glycosyl donor.

To broaden the donor specificity of DSPs, we have applied directed evolution on the cellobiose phosphorylase from *Cellulomonas uda*. This enzyme was chosen because a switch towards galactosyl phosphate as donor could easily be identified by selection on a minimal medium with lactose as the sole carbon source. After one round of errorprone PCR and site-saturation mutagenesis of the hotspots, a double mutant was constructed that is able to phosphorolyse lactose with an activity corresponding to about 10% of the wild-type activity on cellobiose. Such a lactose phosphorylase is not available in nature and further optimization of its activity is currently performed.

P 17

Phosphotriesterase-catalyzed Hydrolysis of Sarin: QM/MM Simulation of the Reaction Pathway

Edyta Dyguda-Kazimierowicz,[1] Jolanta Zurek,[2] Adrian Mulholland,[2] W. Andrzej Sokalski[1] and Jerzy Leszczynski[3]

1 Institute of Physical and Theoretical Chemistry, Wroclaw University of Technology, Wroclaw, Poland, edyta.dyguda@pwr.wroc.pl
2 Centre for Computational Chemistry, School of Chemistry, University of Bristol, Bristol BS8 1TS, UK
3 Computational Center for Molecular Structure and Interactions, Jackson State University, Jackson, Mississippi, USA

Degradation of organophosphorus pesticides and related chemical warfare agents is currently a matter of urgency. Due to broad substrate specificity and high catalytic efficiency, phosphotriesterase (PTE) is among the most studied enzymes capable of biodegradation of neurotoxic organophosphorus compounds. However, experimental data are still insufficient to evaluate the molecular basis of PTE action.

PTE-catalyzed hydrolysis of phosphotriesters is known to proceed upon inversion of configuration at the phosphorus atom according to an in-line displacement mechanism. Presumably, the reaction is triggered by the hydroxyl group bridging the two zinc ions within PTE active site. As argued recently, this role could also be served by the terminally bound hydroxide/water molecule. It is then the nucleophilic attack of the hydroxide on the phosphorus atom that initiates the hydrolysis, followed by the breakage of phosphorus-ester bond and expulsion of the leaving group in an anionic form. In case of sarin, the bond being cleaved is the P-F bond and, accordingly, fluoride constitutes the leaving group. The overall process might also be augmented by zinc ion-substrate interaction which allows for polarization of the phosphoryl oxygen bond and the increase in phosphorus atom electrophilicity. To verify these hypotheses, hybrid quantum mechanical and molecular mechanical (QM/MM) methodology will be employed in theoretical modeling of a potential energy surface for the PTE-catalyzed reaction. PTE-sarin complex derived from classical molecular dynamics will be utilized as a starting point. Apart from determination of the most probable reaction pathway including PTE involvement in a catalytic process, revealing the actual way PTE achieves its enormous rate enhancement would allow for further tailoring of the enzyme activity toward biodegradation of novel substrates.

P 18

Engineering Proteins for Enhanced Activity

Steven Fairchild

The MITRE Corporation, MS H205, 7515 Colshire Drive, McLean, VA, USA, sfairchild@mitre.org

The ability to computationally re-engineer proteins for enhanced binding activity would enable rapid development of therapeutic agents. For example, enzymes could be engineered to have increased affinity towards a reaction's transition state. This would facilitate the development of enzymes with increased and/or novel catalytic activity. The software would also enable the construction of proteins that have improved binding characteristics towards target molecules. These proteins could then be utilized for bio-remediation purposes or as signaling molecules that elicit specific cellular responses.

Along these lines, our group has developed a protein design pipeline (PDP) that facilitates computational re-engineering of protein structures to enhance binding capabilities. The software starts with a known protein structure and a list of residues that constitute the active/binding site. The software then utilizes a genetic algorithm to detect optimal mutations for improving binding activity towards a target molecule (either a reaction's high-energy transition state or a particular molecule of interest). Ultimately, the PDP is shown to work well at optimally re-engineering protein structures to have novel functionality.

P 19

The Novel Substrate Lineages by Key Motif Directed Module Recombination

Le Gao, Xiaoli Zhou and **Yan Feng**

Key Laboratory for Molecular Enzymology and Engineering, Jilin University, Changchun, China, yfeng@mail.jlu.edu.cn

Lipases, esterases and peptidase are important members of serine hydrolase family, which have a conserved C-terminal domain with alpha/beta hydrolase fold. The main difference of them lies on the substrate specificity. Discovery of the substrate specificity mechanism and alteration of the substrate lineages among them became an attractive issue. Here, we construct a novel esterase substrate linkage of the thermophilic aminopeptide hydrolase from *Aeropyrum pernix* K1 (apAPH) by domain recombination evolution in vitro with the enzymes having sequence homology less than 20%. The N-terminal domains ("lip" or "lid") of the enzymes with different substrate specificity profiles were prudently spliced to the catalytic domain of apAPH through domain recombination evolution in vitro and significant changes in substrate specificity profiles were conferred. Distinguished from the parental promiscuous enzyme apAPH which show a maximal activity for p-nitrophenyl octate and Ac-Leu-p-Nitroanilide, the recombinant chimera with "lid" domain from mesophilic *Candida rugosa* lipase lip1, which originally prefers long-chain acyl esters, obtained an ability to hydrolyze long-chain esters at 90°C. Its ratio of the catalytic efficiency (k_{cat}/K_m) between pNP-palmitate and pNP-caprylate was ~13 times higher than apAPH. While the chimera with "cap" domain from thermophilic *Archaeoglobus fulgidus* esterase AFEST, which showed the highest activity towards short-chain acyl esters, preferred short-chain esters. Its ratio of the catalytic efficiency (k_{cat}/K_m) between pNP-butanoate and pNP-caprylate was ~32 times higher than apAPH. Circular dichroism spectra revealed that the chimeras formed the stable structure similar to apAAP. The heat-denaturation curves also showed that the chimeras remained high thermostability. The microenviroment of the active sites of chimeras are varied observed by pH-activity behaviors. The above work showed that the domain recombination evolution in vitro is an important approach for generating the novel substrate linkage.

P 20

Steered Molecular Dynamics and GRID for Simulation of Lipase Activation

Valerio Ferrario,[1] Paolo Braiuca,[1,2] Lorena Knapic,[1] Cynthia Ebert,[1] Lucia Gardossi[1]

1 Department of Pharmaceutical Science, Università degli Studi di Trieste, Piazza le Europa 1, 34127 Trieste, Italy, vferrario@units.it
2 SPRIN Srl, c/o Università degli Studi di Trieste, P.le Europa 1, 34127 Trieste, Italy

Lipases, one of the most widely employed enzyme class, share some basic structural features, such as the presence of a flexible domain, called "lid", determining two different enzyme states. In the presence of a hydrophobic environment the lid changes its conformation, opening the access to the active site, while in polar environments it assumes a closed conformation, covering the active site entrance. While this mechanism is a common feature of most lipases, very little knowledge about the mechanisms of lid mediated activation and the differences between the different lipases has been gathered so far.

Here we present a computational study aimed at understanding the basic mechanism of lipase activation/deactivation by lid opening/closure. The GRID[1] analysis of enzyme surface has been applied to an ensemble of fifteen lipases and it allowed to understand their physical-chemical character in the open and closed conformations. All the lipases show a distinct difference in term of hydrophobic character of the active site in the two conformations, with hydrophobicity prevailing significantly in the open form, while the zone of the enzyme's surface opposite to the active site is invariably polar. This accounts for the ability of these enzymes to work at water/lipid interface and explains why the increase of polarity of the environment leads to enzyme inactivation by lid closure. This closure mechanism has been simulated by molecular dynamics, to better understand the molecular aspects of this conformational change and point out common traits and differences among the lipases. A specific protocol to reduce computational time has been developed, since MD simulation of a system with explicit solvation can take a very long computational time. A faster simulation has been accomplished by using steered MD,[2] applying a specific force vector on the LID, thus favoring a faster conformational change and reducing up to 10 times the length of simulation. Crystal structures of the enzymes in the open and close conformations have been used when available to validate experimentally the simulation protocol. From the analysis and comparison of the different lipases, some interesting common mechanisms can be identified, but also significant differences emerges.

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P 21

Slonomics – A novel Technology for the Generation of Highly Designed Gene Libraries

Fuhrmann Markus

Sloning BioTechnology GmbH, Department of Genetic Diversity, Zeppelinstr. 4, 82178 Puchheim, Germany, m.fuhrmann@sloning.com

The Slonomics technology is an extremely useful tool for the parallel production of gene variants and large gene libraries. Small Sub-fragments of 15 to 21 bp, displaying e.g. an amino acid exchange or a deletion mutation, can be created individually by the successive ligation of triplet based building blocks. These sub-fragments can then be combined with unmodified wild-type fragments in different assembly strategies, resulting in the simultaneous production of many related variants.

This synthesis strategy also allows for a precisely controlled synthesis of even highly diverse SlonoMax mutant libraries: each DNA building block introduces precisely one triplet base pair to a gene sequence, comprising e.g. the codon for a specific amino acid. By adding defined mixtures of building blocks during the synthesis process, multiple amino acid exchanges can be introduced in parallel, also at many consecutive positions of a gene sequence. Since the composition of such a mixture of building blocks is freely definable, codon triplets for up to twenty amino acids can be introduced in pre-determined ratios, with the synthesis of all constant sequence parts still following standard methods.

A mutant library of a green fluorescent reporter gene with modified N-termini only based on silent mutations was created. It was cloned into an inducible expression vector to monitor effects on expression levels of the synthetic gene variants in *E. coli*. The effect of using different codons for synthetic gene variants in expression studies and screening applications will be discussed.

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P 22

Directed Evolution and Mechanistic Studies of Serum Paraoxonases

Leonid Gaidukov, Amir Aharoni and Dan S. Tawfik

Department of Biological Chemistry, Weizmann Institute of Science, Rehovot 76100, Israel, leonid.gaydukov@weizmann.ac.il

Serum paraoxonases (PONs) comprise a family of mammalian hydrolases that exhibit a wide range of promiscuous activities, including organophosphate (OP) detoxification. Human PON1 is associated with high-density lipoprotein (HDL) and plays a key role in the prevention of atherosclerosis. However, the native substrate(s), mechanism of action and the mode of HDL binding of PONs are not known. The objectives of this study are: (i) to investigate PON1's interaction with HDL, and the enzymology of HDL-anchored PON1; (ii) to apply directed evolution on PON1 with the aim of generating novel variants with improved activity and specificity, and thereby studying the structure and function of PON1's active site, and the role of functional promiscuity in enzyme evolution.

We investigated the mode of PON1-HDL interaction by determining the binding affinity, stability, and a range of enzymatic activities, for PON1 interacting with reconstituted HDL particles of different apolipoprotein composition. Our results indicate a unique behaviour of apoA-I particles: these exhibit the highest affinity to PON1 (>109 M-1), and shift PON1 towards a single, highly stable conformation that exhibits high catalytic efficiency and selectivity towards lactone substrates. Altogether, it appears that PON1 evolved as a potent, interfacially-activated lactonase. Future studies will elucidate whether these interfacial effects are mediated through direct interaction of apoA-I with PON1, and which molecular elements of both proteins are involved in this interaction.

Next, PON1 was evolved for increased activity with four substrates representing promiscuous reactions of PON1: i.e. phosphotriesterase, lactonase, esterase and lipase-like. Gene-libraries were prepared by random mutagenesis and screened against each of the target substrates. Three rounds of shuffling and screening led to isolation of specialized PON1 variants with improved activities (>100-fold) and dramatically altered substrate specificities (>10,000 fold). Dramatic shifts in the promiscuous activities of the evolved clones were observed, whereas the native lactonase activity remained almost unchanged. These results demonstrated the unusual plasticity of promiscuous activities versus the robustness of the native function. Mutations found in the evolved clones also defined a set of residues that govern PON1's reactivity and substrate selectivity, allowed us to identify PON1's active site and to obtain insights into how the substrate selectivity of the PON family members evolved in nature. Overall, this study demonstrates that PON1 provides an ample starting point for the evolution of tailor-made hydrolases with improved activity and specificity, and opens the road for the engineering of novel variants with enhanced detoxifying and antiatherogenic properties.

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(Un)Predictable Effects of Distant Mutations on Protein Activity and Stability

Pietro Gatti-Lafranconi, S.M. Caldarazzo, L. Alberghina and M. Lotti

Department of Science, Biotechnology and Biosciences, University of Milano-Bicocca, Piazza della Scienza 2, 20126 Milan, Italy, pietro.gattilafranconi@unimib.it

The biotechnological potential of cold-adapted enzymes is increasingly exploited in industrial application. The major advantages envisaged in the use of enzymes from psychrophylic organisms concern the possibility of carrying out processes at low temperature which, for example, preserve labile compound of nutritional value. However the associated thermolability is a hurdle for application in even moderate conditions or for prolonged times.[1] Our work was focussed on the lipase produced by the bacterium *Pseudomonas fragi* (PFL) which is cold-adapted and thermosensitive.[2] This enzyme was targeted by an approach of directed evolution, i.e. random mutagenesis followed by the screening for improved stability. A further constraint, equal or higher activity at low temperature allowed for selecting only variants were stability was not detrimental for protein flexibility. Two rounds of mutagenesis/selection process provided a small number of PFL variants endowed with higher stability but also with a broader temperature optimum. As a first result, we could conclude that our screening system has been able to identify mutants with both desired properties and that we effectively increased thermal stability, with minor effects on cold-adaptation. Uncoupling of cold-activity and thermolability can be of practical interest, besides providing important information about the role of specific molecular features of the protein.[3] However, detailed analyses on reaction kinetics revealed an apparent increase in efficiency when some mutants were exposed to temperatures already prohibitive for wild type. Further studies required boosting protein production through optimization of a new in vivo system, production of mutants bearing only selected mutations and a more detailed characterization based on inactivation kinetics, specific activity at different temperatures and structural data. Results made us conclude that temperature optimum and thermal-stability have been independently modified by different point mutations. Interestingly, several stabilizing amino acid substitutions mapped into flexible protein regions, in particular in the region known as “the lid” a surface structure that covers the active site and therefore regulate the access of substrates. These results fitted very well with previous studies of rational mutagenesis guided by the comparison with sequence and structure of homologous but more thermostable lipases.[4] To conclude, although temperature adaptation in (extemophilic) enzymes still remains difficult to predict and modify, we gained important information that will require molecular-level understanding to be fully available for practical applications.

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P 24

Illuminating the Mechanism of a PLP-dependant Aldolase Using an *in vivo* Selection System

Lars Giger, Miguel D. Toscano and Donald Hilvert

Department of Chemistry, Laboratory of Organic Chemistry, ETH Zurich, Wolfgang-Pauli Strasse 10, HCI, F336, 8093 Zurich, Switzerland, lars.giger@org.chem.ethz.ch

Directed evolution exploits high-throughput screening or selection systems to rapidly identify enzyme variants with useful properties. In vivo selection systems are particularly powerful in this respect, allowing the simultaneous evaluation of up to 107 variants in a single experiment. We developed a selection system for threonine aldolase activity using a bacterial strain that is auxotrophic for glycine. Growth of this strain can be provided by the addition of β-hydroxyamino acids like L-Thr or L-allo-Thr and the production of an enzyme that can catalyze retro-aldol cleavage of this substrate to glycine. As a test case we constructed a library on the type I L-threonine aldolase from *Caulobacter crescentus* (Cc-LTA). In which four putative active site residues (His91, Asp95, Glu96, and Asp176) were simultaneously randomized to assess their importance for catalysis. Based on sequence analysis of selected clones we found that all four positions are sensitive to substitution, although to varying degrees, ranging from His91, which is absolutely conserved to D95, which can accept a variety of substitution and maintain >5% of activity. Together, the mutagenesis and selection help do define the roles of each residue, which provides deeper insight into catalysis by type I PLP-dependant enzymes, generally. In further experiments, this selection system could be applied to the directed evolution of tailored aldolases for various applications

P 25

Molecular Mechanisms of Glucosyl Transfer in Retaining Disaccharide Phosphorylases

Christiane Goedl,[1] Alexandra Schwarz,[1] Mario Mueller,[1] Lothar Brecker[2] and Bernd Nidetzky[1]

1 Institute of Biotechnology and Biochemical Engineering, Graz University of Technology, Austria, christiane.goedl@tugraz.at
2 Institute of Organic Chemistry, University of Vienna, Austria

Phosphorylases are carbohydrate processing enzymes that catalyze reversible glycosyl transfer from a saccharide donor substrate to phosphate. Structural and mechanistic studies have led to the classification of known phosphorylases into a main group of glycoside hydrolase-like enzymes (GHs) and a smaller group of enzymes related to glycosyltransferases (GTs). A further distinction can be drawn whether the anomeric configuration in the carbohydrate substrate is retained or inverted in the sugar 1-phosphate product. Retaining phosphorylases are found in family GH-13 (sucrose phosphorylase, SPase) and glycosyltransferase families GT-4 (trehalose phosphorylase, TPase) and GT-35 (glycogen, starch and maltodextrin phosphorylase). While the two disaccharide phosphorylases catalyze highly analogous substrate transformations there exists experimental evidence that they have evolved strikingly different reaction coordinates to achieve catalytic efficiency and stereochemical control.[1] Almost four decades ago, elegant biochemical studies revealed that the SPase catalyzed reaction proceeds via the formation of a covalent beta-glucosyl-enzyme intermediate in a two step double displacement-like mechanism.[2] Unlike SPase whose Ping-Pong kinetic mechanism reflects this enzymatic reaction in two catalytic steps, TPase promotes catalysis from a ternary enzyme-substrate complex supporting an internal return-like mechanism.[3] Mutagenesis studies suggest a putative active-site architecture for TPase that is typical of retaining glycosyltransferases of fold family GT-B and markedly different from that of Spase.[4] The model of the active site of TPase serves as point of departure for analyzing the retaining glycosyltransferase-like catalytic mechanism. For the first time, we report on different catalytic functions for residues in the RXXXXK-motif at the phosphate binding site in Tpase.[5] Furthermore, evidence from efforts of mechanism-based redesigning of the catalytic center is presented.

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P 26

QM/MM Investigation of the Reactions of the Hepatitis C virus NS3 Protease with its Main Natural Substrates. Reaction Mechanism and Molecular Dynamics Simulations

Alejandro Rodríguez, Carolina Oliva and **Miguel González**

Departament de Química Física i IQTC, Universitat de Barcelona,
C/ Martí i Franquès 1, 08028 Barcelona, Spain, miguel.gonzalez@ub.edu

The reaction mechanism of the HCV NS3 protease with the NS4B/5A and NS4A/4B substrates was studied using the AM1/CHARMM27 Quantum Mechanics/Molecular Mechanics hybrid approach. The main goal of this work is to try to understand the differences found between the reaction mechanism of each substrate and the role played by the different enzymatic residues. This knowledge will hopefully be of interest in the development of new NS3 protease inhibitors based on transition state analogs. The two first steps of the mechanism were examined here (acylation step and breaking of the peptide bond) and barrier energies at the AM1/CHARMM27 level were corrected by applying the MP2 ab initio method. The acylation step is the rate limiting step and occurs through a tetracoordinated intermediate, as previously suggested for other Serine proteases. Specificities in the mechanism of NS4B/5A were attributed to the presence of a Proline residue in the substrate P2 position. The structural and energetic analysis confirmed the importance of the oxyanion hole in the electrostatic stabilization of the tetracoordinated intermediate. The role of other residues, e.g., Arg-155 and Asp 79, and the viability of Arg-155 mutants and its resistance to some protease inhibitors was also explained. Finally, molecular dynamics simulations using the Umbrella Sampling were also performed to determine via the WHAM technique the free energy profiles for the acylation and peptide bond breaking steps. All the results obtained and, in particular, the comparison between the purely geometric and the dynamics results will be presented at the meeting.

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P 27

Iterative Saturation Mutagenesis Based on B-factors to Increase Thermostability of *Aspergillus niger* Epoxide Hydrolase

Manfred T. Reetz and **Yosephine Gumulya**

Max-Planck-Institut of Coal Research, Kaiser-Wilhelm-Platz 1,
45470 Mülheim an der Ruhr, Germany, gumulya@mpi-muelheim.mpg.de

In the last few decades enzymes start to play a significant role in chemical industry. However most of enzymes are less stable in harsh chemical reaction environment. Hence lots of methods were developed in order to improve enzyme stability, especially thermostability. Vihinen (1987)[1a] and Parthasarathy and Murthy (2000)[1b] correlated the increasing protein thermostability with the overall decreasing protein flexibility. Based on this, our group has performed recently an Iterative Saturation Mutagenesis (ISM) to improve protein thermostability using B-factors (atomic displacement parameters obtained from X-ray data).[2,3] With B-FIT (B-factor iterative test) only those amino acids that display the highest B-factors are targeted.[2,3] B-FIT has been successfully applied to *Bacillus subtilis* lipase A (BSL, 19kDa).[2,3] However, we would like to extend its application to a bigger protein. For this purpose, we choose *Aspergillus niger* epoxide hydrolase (AnEH, 44 kDa).

More than 50 mutants were obtained from library A (Ala 321 Ser 322), with ΔT50 1-6°C higher than that of WT. Library A is located in part of unresolved region in AnEH crystal structure, residue 320-328, which is supposed to have the highest B-factors. Hence our result showed that B-FIT also works to increase thermostability of bigger protein. The highest T5015 achieved so far is 56.5°C and 56.1°C, however in order to complete the iterative cycles of saturation mutagenesis, further rounds of mutagenesis still have to be performed.

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P 28

Improvement of Protein Thermostability, Solubility and Enzymatic Activity by Directed Evolution

Jayme Williams and **Volker Heinrichs**

Athenix, 108 T.W. Alexander Drive, Research Triangle Park, 27709, NC, USA, vheinrichs@athenixcorp.com

Protein solubility and thermostability are important factors in determining the availability and activity of many, if not most, proteins. We have used directed evolution to improve the thermostability and solubility of members of two different classes of proteins; EPSPS enzymes and insecticidal Cry endotoxins.

The enzyme 5-enolpyrovoylshikimate 3-phospate synthase (EPSPS) catalyses the conversion of phosphoenolpyruvate and shikimate 3-phosphate to 5-enoylpyrovoyl-shikimate-3-phosphate. EPSP synthase is part of the shikimate pathway and is essential for the synthesis of aromatic amino acids in plants and microbes. EPSPS are inhibited by the herbicide glyphosate, and the use of glyphosate resistant EPSPS in crop plants is of considerable commercial significance. We mutagenized the catalytic center of an EPSPS to improve its thermostability, solubility and glyphosate resistance. Mutants were assayed for solubility using a dot blot assay. Next mutants were incubated for various times at 37°C, a nd the effect of the increased temperature on EPSPS activity was determined using an in vitro enzymatic assay. We obtained an EPSPS variant with 5 fold improved thermostability at 37°C carrying a point mutation i n the catalytic center. This EPSPS variant also possesses a 7 fold increased Ki for glyphosate. Cry protein endotoxins are expressed in *Bacillus thuringiensis*, and members of this protein family are toxic to *Coleoptera*, *Lepidoptera*, and nematodes. Crop plants expressing Cry proteins show improved resistance to insects. Cry toxins form insoluble crystals that are solubilized in the insect gut upon ingestion. The solubility of Cry toxins correlates with their insecticidal activity. We used random PCR mutagenesis to improve the solubility of a corn rootworm active Cry toxin. Screening of mutagenized Cry protein variants by dot blot led to the identification of variants with 4 fold improved solubility.

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Engineering a Selective Small-molecule Substrate Binding Site into a Deoxyribozyme

Claudia Hoebartner[1] and Scott K. Silverman[2]

1 AG Nucleic Acid Chemistry, Max-Planck-Institute for Biophysical Chemistry, Göttingen, Germany, claudia.hoebartner@mpibpc.mpg.de
2 Department of Chemistry, University of Illinois at Urbana-Champaign, IL, USA

Most nucleic acid enzymes (ribozymes and deoxyribozymes) catalyze reactions of oligomeric nucleic acid substrates but not small molecules. By rational design, we elaborated a deoxyribozyme into a catalyst that provides a selective binding site for a small-molecule substrate. The 7S11 deoxyribozyme and its variant 10DM24 are intriguing DNA catalysts that synthesize 2',5'-branched RNAs. These deoxyribozymes mediate the nucleophilic attack of a specific internal adenosine 2′-hydroxyl group of one RNA substrate into the 5′-triphosphate of a second RNA substrate. The bulged branch-site adenosine nucleophile and the triphosphate electrophile are positioned at the intersection of a three-helix junction, which has been demonstrated by comprehensive nucleotide covariation analysis. Truncation of the electrophilic RNA substrate by removal of its 5'-terminal nucleotide is shown to generate a platform for molecular recognition of a mononucleotide triphosphate (NTP) via Watson-Crick base-pairing interaction with a specific nucleotide of the deoxyribozyme (the "recognition nucleotide"). The truncated original substrate serves as a cofactor that mediates folding of the deoxyribozyme into the active three-helix junction conformation. A variety of purine NTPs serve as substrates for the modified 10DM24 deoxyribozyme, provided that Watson-Crick complementarity is retained between the substrate and the recognition nucleotide. Higher reaction rates are observed for NTPs that are capable of forming three versus two hydrogen bonds (e.g., guanosine versus inosine, or 2,6-diaminopurine riboside versus adenosine). We have also examined the contributions of ribose functional groups and structural preorganization within the NTP substrate for the 10DM24-catalyzed reaction.

In summary, starting from an in vitro selected deoxyribozyme we have shown that strategic modifications in the vicinity of the active site can be used to design DNA catalysts that have selective binding sites for small-molecule substrates. These results will assist future studies towards the development of deoxyribozymes as improved biocatalysts that utilize small molecules other than nucleic acid monomers.

P 30

Designing *de novo* Superoxide Dismutases

Olga C. Iranzo

Instituto de Tecnologia Química e Biológica (ITQB), Estação Agronómica Nacional, Av. da República, 2780-157 Oeiras, Portugal, oiranzo@itqb.unl.pt

Defining how one molecule recognizes and controls the properties of another molecule or ion is an important issue in chemistry. Metalloproteins are proficient in controlling the properties of their metal ions by controlling the geometries, coordination spheres and type of ligands bound to them. This control is crucial for proper biological activity. Iron- and manganese-containing Superoxide Dismutases (Mn-SDOs and Fe-SODs) are metalloenzymes that play a key role in cellular protection against oxidative stress conditions by catalyzing the dismutation of superoxide radicals ($O_2^{\bullet}$) into the less toxic dioxygen and hydrogen peroxide. Within the Mn/Fe SOD family, there is a high degree of structural homology and the transition metal cofactor (Mn or Fe) is coordinated by the same set of amino acids: two histidines and an aspartic acid in the equatorial plane, and a histidine and a solvent molecule in the axial positions forming a distorted trigonal bipyramidal geometry. Nonetheless, Mn-SODs are not active with Fe and neither are the Fe-SODs with Mn. Intriguingly, the cambialistic SODs function with either of the two metals. This functional selectivity has fascinated chemists and biochemists and a lot of effort has been devoted to its understanding by either studying the natural system or small synthetic inorganic models. Our approach is to use the alternative route of the de novo design of metallopeptides to generate peptidic models of Mn/Fe SODs. A stable de novo peptidic scaffold was used as a starting point to introduce the active center of the Mn/Fe SODs and different mutants were generated. UV-Vis, EPR and CD spectroscopies are now being used to study Fe and Mn binding. The study of the SOD activity of these constructs will give us crucial insights into the functional selectivity that the natives enzymes have.

P 31

Dynamics of water molecules at the tunnel mouths of haloalkane dehalogenases studied by time resolved fluorescence spectroscopy and computer simulations

Andrea Jesenska,[1] Jan Sykora,[2] Agnieszka Olzynska,[2] Jan Brezovsky,[1] Zbynek Zdrahal,[3] Martin Hof,[2] Jiri Damborsky[1]

1 Loschmidt Laboratories, Faculty of Science, Masaryk University, Kamenice 5/A4, 625 00 Brno, Czech Republic, andreaj@chemi.muni.cz
2 J. Heyrovsky Institute of Physical Chemistry of the ASCR, v. v. i., Dolejskova 3, 182 23 Prague 8, Czech Republic
3 Department of Functional Genomics and Proteomics, Institute of Experimental Biology, Faculty of Science, Masaryk University, Kamenice 5/A2, 625 00 Brno, Czech Republic

Haloalkane dehalogenases (EC 3.8.1.5) are microbial enzymes that cleave a carbon-halogen bond in broad range of halogenated compounds. Active sites of haloalkane dehalogenases are buried between α/β hydrolase fold domain and a cap domain. The openings of tunnels which connect the active sites with the protein surface are evolutionally the most variable regions in the structures of haloalkane dehalogenases. Tunnels serve as the transport routes for the substrates and products. Dynamics of solvent molecules may have impact on binding affinities and the catalytic rates and it is therefore of great importance to better understand how this dynamics differs among different proteins.

In this study, the dynamics of water molecules at the tunnel mouth of haloalkane dehalogenases DbjA and DhaA was monitored by means of time resolved fluorescence spectroscopy and molecular dynamic simulations. Reaction mechanism of dehalogenation involves nucleophilic attack of the carboxylate oxygen of an aspartate group on the carbon atom of the substrate, yielding displacement of the halogen as halide and formation of a covalent alkyl-enzyme intermediate. The alkyl-enzyme intermediate is subsequently hydrolyzed by a water molecule which is activated by the catalytic histidine. We took the advantage of the covalent labelling of haloalkane dehalogenases carrying mutations in catalytic histidine with the fluorescent coumarin dye and adapted a protocol, which specifically labels the tunnel mouths with simultaneous elimination of all unbound and non-specifically bound dye molecules.

The measurements of time-resolved fluorescence anisotropy and time resolved emission spectra with carefully prepared protein-due complexes showed significant differences in the polarity, accessibility and mobility of the dye and its microenvironment for two studied enzymes. Coumarin bound in haloalkane dehalogenase DbjA is more flexible and its microenvironment is more polar in comparison with coumarin bound in DhaA. The dye in DbjA displays higher hydration whereas solvent around coumarin attached to DhaA is more viscose. The obtained experimental data were consistent with the results obtained by molecular dynamics calculations and reflect anatomy of the tunnel mouths seen in the crystal structures. Additional studies with natural and engineered dehalogenases, currently ongoing in our laboratories, are needed for better understanding of the importance of tunnel mouths' dynamics for functional properties of the enzymes with buried active sites.

P 32

Converting an Esterase into an Epoxide Hydrolase

Helge Jochens,[1] K. Stiba,[1] C. Savile,[2] R. Fujii,[2] J.-G.-Yuo,[2]
R.J. Kazlauskas[2] and Uwe T. Bornscheuer[1]

1 Institute of Biochemistry, Biotechnology and enzyme catalysis, University of Greifswald, Felix-Hausdorff-Str. 4, 17487 Greifswald, Germany, helge.jochens@uni-greifswald.de
2 University of Minnesota, BioTechnology Institute, Gortner Avenue 1479, St. Paul, MN, USA

The α/β-hydrolase fold is a common structural motif in many hydrolytic enzymes, but the interconversion of one enzyme into another has not been reported yet. This indeed turned out to be very tricky, but careful design of point mutations and the introduction of a loop finally created epoxide hydrolase activity in the esterase scaffold resulting in an enantioselective chimeric enzyme.

P 33

Directed Evolution of Two Key Cellulases for the Conversion of Biomass to Ethanol

Aubrey Jones, Paul V. Harris, Ana L. Fidantsef,[1] Michael Lamsa, Brian Gorre-Clancy, Jung Yi, Connie Ward,[2] Joel R. Cherry and Sarah A. Teter

Novozymes, Inc 1445 Drew Avenue, 95618 Davis, CA, USA, aujo@novozymes.com
1 Arcadia Bio Science, Davis, CA, USA
2 GSK Biologicals Hamilton, MT, USA

Plant biomass, including the cellulosic material comprising cell walls of higher plants, is the most abundant source of fermentable carbohydrates in the world and as of yet, a barely-utilized renewable energy resource. Utilization of this carbohydrate for biofuel (ethanol) depends on our ability to cheaply and efficiently convert the complex carbohydrate into sugar. The conversion of cellulosic biomass to fermentable glucose requires the coordinated action of several enzymes that include endocellulases, exocellulases and ß-glucosidase (BG). The glycosyl hydrolase family 7 cellobiohydrolase I (CBHI) has been shown to be vital in the overall hydrolysis of cellulose as it cleaves cellobiose exo fashion from the reducing termini of cellulose. The importance of CBHI is self evident when considering that the CBHI component alone is about 60% of the total secreted cellulases from the cellulytic fungi *Trichoderma reesei*. Similarly the glycosyl hydrolase family 3 ß-glucosidase also plays an important as it catalyzes the conversion of cellobiose to glucose. The action of ß-glucosidase is critical, as it alleviates end-product inhibition from cellobiose.

As a strategy for improving the effective catalytic rate of these enzymes, we employed directed evolution and rational design, combined with a high-throughput screens for improved high temperature activity (CBH I) and improved residual activity following thermal challenge (BG). By screening for improved residual activity of BG, we discovered variants with dramatically improved thermostability. For CBHI evolution, a tiered screening approach was utilized. First, libraries were screened for improved high temperature activity on a small fluoregenic substrate; we thus achieved a higher specific activity relative to the parent enzyme assayed at its optimal temperature. Selected variants were then screened for effectiveness in hydrolyzing crystalline lignocellulose. When assayed on industrially relevant lignocellulose substrates, the thermally active CBHI variants showed improved high temperature performance when combined with other thermally stabilized components.

P 34

3DM: A tool for Optimal Use of Superfamily Data for Enzyme Engineering

Henk-Jan Joosten

Department of Microbiology, Faculty of Fungal Genomics, Wageningen University and Research Centre, Dreijenplein 10, 6703HB Wageningen Gelderland, The Netherlands, henk-jan.joosten@wur.nl

A powerful method to gain biological insights in the functioning of a protein is to use data available for the protein (super) family and transfer this data to the protein of interest. Systematically correlating the sheer amount of data available is difficult and time consuming. Therefore, protein superfamily-specific databases are needed. It is shown that such systems can help to predict protein interaction sites, active site residues, residues important in enzyme specificity/activity, protein stabilizing residues, etc. These systems are therefore powerful tools in drug design and protein engineering studies, but building and keeping such systems op to date is time consuming.

Here we describe 3DM, a system that can automatically build such protein superfamily databases. The system starts with building a large accurate structure based multiple sequence alignment of a class of proteins (superfamily). Secondly, it collects and stores amino acid associated data, such as mutational information, ligand- and substrate contacts, etc. together with data derived form the alignment such as correlated mutations and conservation information. These information types are linked to the alignment making it possible to easily transfer information from well studied members of the family to the protein of interest. Navigating between the alignment and associated data is done via interactive HTML pages. A 3DM built for the nuclear receptor superfamily predicted a role in cofactor binding for 15 amino acid positions. A literature search revealed that mutating eleven of these positions indeed has an effect on cofactor binding. Two of the remaining four are located in the cofactor binding site, suggesting a direct role in cofactor binding and mutating a third residue indeed showed a profound effect on cofactor binding.

The PEP/ICL enzyme superfamily 3DM database predicted an important function for an oxaloacetate hydrolase subfamily specific serine. Site directed mutagenesis of this serine changed substrate specificity which revealed the unknown reaction mechanism. The cupin superfamily 3DM database was used to predict that a specific double mutant could compensate for the loss of activity that remarkably resulted from a single mutant located in a loop on the outside of the protein. Construction of the double mutant resulted in an enzyme with twice the activity compared to the wildtype enzyme.

P 35

Directed Evolution of *in silico* Designed Enzymes

Olga Khersonsky

Department of Biological Chemistry, Weizmann Institute of Science, Ullmann building, Room 207, 76100 Rehovot, Israel, olga.khersonsky@weizmann.ac.il

Directed evolution has proven a powerful method for generating novel catalysts. However, an almost absolute requirement of directed evolution is a starting point with some initial activity, which can be either a natural enzyme with a weak promiscuous activity. More recently, the power of computational design for the engineering of novel proteins has also been demonstrated, thus providing intriguing opportunities for directed evolution.

In silico designed templates were used as starting points for directed evolution of Kemp eliminases. Kemp elimination is a model reaction of proton transfer from carbon, and since it has no biological relevance, no natural enzyme catalyses it efficiently. Artificial enzymes with weak Kemp elimination activity were designed in the laboratory of Prof. David Baker (University of Washington, Seattle, USA), by grafting the side chains necessary for efficient catalysis of Kemp elimination onto an existing TIM-barrel scaffold. Three designed starting points were subjected to rounds of mutation and selection, in order to evolve an efficient catalyst for Kemp elimination. In case of the enzyme KE7, after seven rounds of mutation and selection, >200-fold increase in activity was obtained, and the evolved variants were characterized structurally and biochemically. The enzyme KE59 was first stabilized by incorporation of "back to consensus" mutations, and its directed evolution is currently in progress. I will describe the laboratory evolution process, and how the evolved mutations fine-tune the catalysis in these novel catalysts.

P 36

Pathways and Mechanisms for Product Exit and Water Exchange in Engineered Haloalkane Dehalogenase DhaA Explored using Classical and Random Acceleration Molecular Dynamics Simulations

Martin Klvana,[1] Martina Pavlova,[1] Tana Koudelakova,[1] Radka Chaloupkova,[1] Pavel Dvorak,[1] Alena Stsiapanava,[2] Michal Kuty,[2] Ivana Kuta Smatanova,[2,3] Jan Dohnalek,[4] Petr Kulhanek,[5] Rebecca C. Wade,[6] and Jiri Damborsky[1]

1 Loschmidt Laboratories, Institute of Experimental Biology and National Centre for Biomolecular Research, Faculty of Science, Masaryk University, Kamenice 5/A4, 625 00 Brno, Czech Republic, martink@chemi.muni.cz
2 Institute of Physical Biology, University of South Bohemia Ceske Budejovice, Zamek 136, 373 33 Nove Hrady, Czech Republic
3 Academy of Science of the Czech Republic, Zamek 136, 373 33 Nove Hrady, Czech Republic
4 Institute of Macromolecular Chemistry, Academy of Sciences of the Czech Republic, Heyrovskeho namesti 2, 162 06 Praha 6, Czech Republic
5 Laboratory of Computational Chemistry, National Centre for Biomolecular Research, Faculty of Science, Masaryk University, Kamenice 5/A4, 625 00 Brno, Czech Republic
6 EML Research, Villa Bosch, Schloss-Wolfsbrunnenweg 33, D-69118 Heidelberg, Germany

Numerous experimental and computational studies of cytochrome P450s, acetylcholinesterase, haloalkane dehalogenase and other enzymes with buried active site underline importance of active site access pathways in determining substrate specificity and raise intriguing question of how do substrates enter and products leave the active site of an enzyme.

Here, we studied product exit pathways from a rhodococcal haloalkane dehalogenase DhaA and its mutants using classical and enhanced sampling random acceleration molecular dynamics (RAMD) simulations. The virtue of RAMD is to get insight into a process of product exit which can occur on millisecond or longer timescale and is therefore inaccessible by classical molecular dynamics simulation techniques. We used a chloride and 2,3-dichloropropane-1-ol as the products of dehalogenation of a toxic xenobiotic 1,2,3-trichloropropane as the probes. While the chloride exit was observed in classical MD as a fast water-assisted process, RAMD was needed in order to observe slower exit of the alcohol.

One exit pathway (p1) was identified for the chloride and five exit pathways (p1, p2a, p2b, p2c and p3) for the alcohol. p1 was proposed to serve as the main product exit pathway and water exchange pathway; p2a, p2b, p2c and p3 were assigned as auxiliary alcohol exit and water exchange pathways. Natural fluctuations of the pathways were not generally sufficient to allow exit of the product molecules. Widening of p1 and opening of other pathways was induced by a passing ligand and was accomplished through small backbone and side-chain fluctuations (all pathways), gating by aromatic residues (p1 , p2b and p3), and breakage of a beta-bridge interaction (p2c). Moreover, mutations rationally introduced into the structure of DhaA, I135V and W141F, were required to allow access of the alcohol to p2b and p2c, respectively, whereas I135F effectively blocked p2a. Interestingly, C176Y and its combination with V245F, A172F and A172 changed mechanism of p1 opening from permanent to ligand-induced.

Classical MD simulations showed that the bulky substitutions were also responsible for improved shielding of the active site from bulk solvent upon substrate binding, at the same time allowing water to penetrate into the active site after halide formation to aid the product clearance. In conclusion, comparison of the simulations conducted with the wild type and mutant enzymes revealed that substitutions introduced to the access tunnels can change both accessibility and the exchange mechanism.

P 37

3D-QSAR Model for the Quantitative Prediction of Enantioselectivity of Lipase B from *Candida antarctica*

Knapic Lorena,[1] Paolo Braiuca,[1,2] Valerio Ferrario,[1]
Cynthia Ebert[1] and Lucia Gardossi[1]

1 Department of Pharmaceutical Science, Universita' Degli Studi di Trieste, P. le Europa 1, 34127 Trieste, Italy, lknapic@yahoo.com
2 SPRIN Srl c/o Università degli Studi di Trieste, P. le Europa 1, 34127 Trieste, Italy

Computational analysis of enzyme-substrate interaction can provide valuable explanations of enzyme stability and selectivity and therefore be a guiding line to rationalize the design of biocatalysed reaction systems. Recent studies demonstrated that techniques involving multivariate statistical analysis can be used to evaluate and predict quantitatively these important enzyme properties through the elaboration of statistical models able to correlate the 3D structure of molecules with enzyme's properties.

Methods able to predict quantitatively enantioselectivity would have great practical and theoretical impact and would represent an. actual alternative to experimental screening procedures, which are usually time-consuming and expensive. Obtaining a 3D-QSAR model involving this property means working out a new type of approach by introducing a new generation of molecular descriptors i.e. differential molecular interaction fields. A few examples of application of 3D-QSAR to biocatalysis have been reported recently.

Now we present the application and the expansion of this approach l to the achievement of the first quantitative predictive model for the enantioselectivity of *Candida antarctica* lipase B (CALB). In order to allow the mathematical and statistical processing of experimental data largely available in the literature (namely Enantiomeric ratio E) a novel class of GRID-based molecular descriptors, the differential MIFs, have been developed. These descriptors proved to be efficient in providing the structural information which are needed for creating the regression model. On this basis, and multivariate statistical analysis, based on PLS, have been used for the analysis of data available from the literature and for the construction of the first 3D-QSAR model able to predict the enantioselectivity of CALB. Results indicate that the model is statistically robust and predictive.

P 38

Targeted Cellular Delivery of Catalytically Improved Nucleoside Kinases to Enhance Metabolic Activation of Prodrugs and Improve Anticancer and Antiviral Therapy

Manfred Konrad,[1] Theresa McSorley,[1] Arnon Lavie[2] and Stephan Ort[1]

1 Enzyme Biochemistry, Max-Planck-Institute for Biophysical Chemistry, Goettingen, Germany, mkonrad@gwdg.de
2 Biochemistry and Molecular Genetics, University of Illinois at Chicago, Chicago, USA

Nucleoside analogs (NA) are widely used in chemotherapy of cancer and viral infections. These compounds are commonly administered in their unphosphorylated form, the so-called prodrug. NAs cross the cell membrane via nucleoside transporters and are then converted to their 5'-triphosphorylated states (NA-TP) by nucleoside and nucleotide kinases. The intracellular NA-TPs can efficiently terminate synthesis of nucleic acids in viral replication and switch on the apoptotic cascade in cancer cells. The objective of our study is to improve enzyme-prodrug systems that are of potential use in gene therapy approaches. NAs, such as AZT (3'-azido-3'-deoxythymidine) for the treatment of HIV infection, the guanosine analogs acyclovir (ACV) and ganciclovir (GCV) used against Herpes virus, or the anticancer compounds AraC (cytosine arabinoside) and gemcitabine (2′-deoxy-2′,2′-difluorocytidine), are phosphorylated by different kinases. The rate-limiting reaction in metabolic activation is often the first, and in some cases the second phosphorylation step. Based on crystal structure analyses of various enzyme-nucleotide complexes we designed mutants of the human TMP kinase (hTMPK) that phosphorylate AZTMP up to 200-fold faster than wildtype, thus removing the bottleneck in AZT activation. Expression of this enzyme in human cells leads to 10-fold higher intracellular concentrations of AZTTP and to enhanced HIV inhibition. Moreover, we could resensitize drug-resistant HIV to AZT through direct cellular delivery of engineered hTMPK. Recently, we have evaluated this enzyme/prodrug combination as a novel means to efficiently induce cell death in primary and immortal T cells and to kill erythroid leukemia cells in a mouse tumor model. Secondly, an engineered human deoxycytidine kinase variant catalyzes the activation of the prodrugs AraC and gemcitabine more efficiently than the natural enzyme. Remarkably, this enzyme is highly active in phosphorylating NAs of the non-physiological stereochemical configuration (L-enantiomer), such as 3TC (lamivudine) and TRO (troxacitabine), that are less toxic in vivo and biologically more potent than the corresponding D-enantiomers. Thus, our work highlights the concept of a gene or enzyme-based therapeutic treatment involving expression or direct intracellular protein transduction of catalytically improved human enzymes. In future applications, suicide genes may become an even more promising tool to establish control over the fate of cells transduced with integrating viral vectors, and pave the way to the development of novel schemas in nucleoside prodrug-dependent cancer chemotherapy.

P 39

Modification of Activity and Enantioselectivity of an Epoxide Hydrolase by Saturation Mutagenesis, and Novel Epoxide Hydrolases from Environmental DNA

Michael Kotik, Vaclav Stepanek and Helena Maresova

Institute of Microbiology, Academy of Sciences of the Czech Republic, Videnska 1083, 142 20 Prague, Czech Republic, kotik@biomed.cas.cz

Saturation mutagenesis at the entrance to the substrate tunnel of the epoxide hydrolase from *Aspergillus niger* M200 resulted in enzyme variants with in several cases profound differences in activity and enantioselectivity towards various epoxides when compared to the data of the wild-type enzyme. Further, we describe a convenient method for amplification of novel epoxide hydrolase-encoding genes directly from the metagenome. In a first step, small specific regions of putative epoxide hydrolase genes were amplified by using PCR with degenerate consensus primers specific for prokaryotic epoxide hydrolases, and environmental DNA as template. In a second step, the sequences obtained from randomly selected epoxide hydrolase gene fragments served as the starting point for genome walking PCR. This technique enabled us to recover complete novel epoxide hydrolase genes from extracted environmental DNA. The genes were cloned and overexpressed in *Escherichia coli*. The recombinant enzymes were characterized in terms of their substrate range, enantioselectivities and regioselectivities.

P 40

Analysis of Substrate Specificities of Wild-type Haloalkane Dehalogenases and their Mutants by Multivariate Statistics

Tana Koudelakova,[1] Eva Chovancova,[1] Martina Pavlova,[1] Marta Monincova,[1] Tomas Mozga,[1] Andrea Jesenska,[1] Khomaini Hasan,[1] Pavel Dvorak,[1] Jiri Jarkovsky[2] and Jiri Damborsky[1]

1 Loschmidt Laboratories, Institute of Experimental Biology and National Centre for Biomolecular Research, Faculty of Science, Masaryk University, Kamenice 5/A4, 62500 Brno, Czech Republic, tangerine@chemi.muni.cz
2 Institute of Biostatistics and Analyses, Faculty of Medicine and Faculty of Science, Masaryk University, Kamenice 126/3, 62500 Brno, Czech Republic

Haloalkane dehalogenases (HLDs, EC 3.8.1.5) are broad-specificity enzymes that cleave a carbon-halogen bond in a broad range of chlorinated, brominated and iodinated compounds. Dozen wild-type HLDs from various hosts and a number of their mutants have been characterised so far. With growing number of biochemically characterised HLDs it is becoming important to quantitatively analyse and compare their substrate specificities. Here we conduct such analysis and address several important questions. Can we classify HLDs according to their substrate specificity? Do substrate specificities of HLDs reflect their evolutionary history? Do substrate specificities of wild-type enzymes differ significantly from specificities of mutants constructed by protein engineering?

Firstly, multivariate analysis was conducted on substrate specificity profiles of nine experimentally characterised HLDs and obtained clustering was compared with clustering based on phylogenetic analysis. Secondly, multivariate analysis was carried out for the dataset of nine wild-type and five engineered enzymes. Principal component analysis was conducted using the enzymes as objects and thirty halogenated substrates as variables. Specificity profiles were obtained by mathematical transformation of specific activity data: decimal logarithm was calculated for each activity value and proportion of each logarithmic value on the sum of all logarithmic values for the individual enzyme was calculated. Using this transformation, the effect of overall activity on distribution of enzymes in the multivariate space is eliminated. Phylogenetic analysis was conducted for protein sequences of HLDs aligned by MUSCLE and trees were calculated by PHYML and MLDIST programs using the WAG evolutionary model and parameters estimated by the PROTTEST server.

The multivariate analysis of specificity profiles clustered wild-type enzymes into four groups whereas phylogenetic analysis suggested subdivision of studied HLDs into three distinct subfamilies. Specificity groups of HLDs were formed irrespectively of their membership to individual phylogenetic subfamilies. In other words, no relationship could be established between substrate specificity groups and phylogenetic subfamilies. The analysis conducted with the combined dataset of wild-type and mutant enzymes revealed that substrate specificities of engineered and wild-type enzymes differ, yet all engineered enzymes clustered together with their templates within respective specificity groups. Altogether, the multivariate statistics proved to be an effective tool for analysis of substrate specificity data.

P 41

Protein Engineering of the Cancer Drug: L-Asparaginase

Marc N Offman,[1] **Marcin Krol**,[1] Naina Patel,[2] Vaskar Saha[2] and Paul A Bates[1]

1 Biomolecular Modelling Laboratory, Cancer Research Institute, Lincoln's Inn Fields Laboratories, London, UK, marcin.krol@cancer.org.uk
2 Cancer Research UK Children's Cancer Group,Paterson Institute for Cancer Research, The University of Manchester, Manchester, UK

The bacterial enzyme, L-Asparaginase (L-Asn), is widely used in haematopoetic cancers such as acute lymphoblastic leukaemia (ALL). L-Asn, which is active as a tetramer, hydrolyses the amino-acid asparagine to aspartic acid and ammonia. Lymphoblasts only synthesise small amounts of asparagine; therefore, they are dependent on an exogenous supply. Administration of the drug L-Asn lowers asparagine blood levels and eventually leads to the apoptosis of leukemic lymphocytes.

L-Asn treatment is highly effective and has few side effects compared to standard chemotherapeutics. However, some patients do not respond to the treatment and become allergic to the drug. These side effects, are found primarily in high-risk patients and are caused by the degradation of L-Asn by two endopetidases. The degradation is responsible for rapid inactivation of L-Asn and an increased accessibility of antigenic epitopes.

Using a combination of structure and sequences analysis we identified the primary cleavage site. Experimental verification confirmed that a glycine mutation at this position result in a cleavage-resistant enzyme, albeit with a decreased activity. Further studies, employing genetic algorithms (GA) to search the sequence space at the primary cleavage site, combined with MD simulations, were applied to recover WT activity for a cleavage-resistant mutant. This resulted in the design of cleavage-resistant mutants with an increased activity of 23% compared to the WT.

Furthermore, we increased the stability of the L-Asn active tetramer, by the introduction of stabilising mutations at the monomer-monomer interface. Pre-selection of mutants was based on the amino-acid sampling frequencies calculated using our GA search engine. Mutants with the highest frequencies were subjected to free-energy of binding calculation; these free energies were used to select the final mutations. Preliminary experimental data confirmed our computational predictions and showed that a single point mutation at the interface can increase the activity by up to 200%. Ultimately, we intend to combine the best mutations in order to create a cleavage-resistant drug with a markedly increased activity and stability compared to the WT. This will enable therapy at a lower dosage, thus reducing the antigenic response to the drug.

P 42

Mechanistic Studies and Modification of Prenylating Enzymes

Julia Kufka, L. Bräuer, N. Günnewich and L.A. Wessjohann

Department of Bioorganic Chemistry, Leibniz-Institute of Plant Biochemistry, Weinberg 3, 06120 Halle/Saale, Germany, wessjohann@ipb-halle.de

Prenyl-converting or -transfering enzymes are responsible for the formation or modification of over 70.000 naturally occurring isoprenoid-containing compounds. Approximately half of them are terpenoids, while the other half are chimeric compounds of moieties from other biosynthetic origins coupled to isoprenoids (meroterpenoids). All organisms possess essential isoprenoids. The biosynthesis of this huge class of compounds is of great interest not only for biochemists and biologists, but also for medicinal and organic chemists. Isoprenoids exhibit antimicrobial, anti-inflammatory, antiviral, and anticancer activity, with Paclitaxel (Taxol) as one prominent example. The synthesis of terpenoids can be complicated and usually requires a multitude of steps. Biosynthetic enzymes are rarely used in such syntheses but in principle they can cover some of these steps if applied as biocatalysts on suitable precursors. They might subsequently be improved by applying structure-based rational protein design, targeted mutagenesis, or directed evolution. The aim of the present study is to understand the mechanisms of prenylating enzymes by substrate studies, bioinformatics and computational chemistry, and site-directed mutagenesis, and to modify them in order to increase desirable properties of the biocatalysts, e.g. changing the product selectivity or the stability.

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P 43

A Structured-controlled Lipase Enantioselectivity Investigated by a Path Planning Approach

Vincent Lafaquière,[1,2,3] S. Barbe,[1,2,3] S. Guenot,[1,2,3] D. Guieysse,[1,2,3] J. Cortés,[4] T. Siméond, P. Monsan,[1,2,3] I. André,[1,2,3] and M. Remaud-Siméon[1,2,3]

1 Laboratoire d'Ingénierie des Systèmes Biologiques et des Procédés, INSA, UPS, INP, Université de Toulouse, 135 Avenue de Rangueil, F-31077 Toulouse, France, vincent.lafaquiere@insa-toulouse.fr
2 Ingénierie des Systèmes Biologiques et des Procédés, UMR792, INRA, F-31400 Toulouse, France
3 CNRS, UMR5504, F-31400Toulouse, France
4 LAAS-CNRS, 7 av. Du Colonel-Roche, 31077 Toulouse, France

A great challenge for pharmaceutical and industrial chemistry of the next decades is the discovery and development of fast and economic deracemization processes. Among the existing enantioselective processes, biocatalysis based on the enzymatic resolution of racemates appears as a method of choice. In particular, lipases are among the most employed catalysts in organic synthesis to catalyze kinetic resolution of a wide range of substrates yielding optically pure compounds. However, few studies aiming at understanding better the factors controlling enantioselectivity of these enzymes at a molecular level have been reported.

Within this context, our work has been focused on the understanding of the *Burkholderia cepacia* lipase (BCL) enantioselectivity towards 2-substituted acids which are important chiral building blocks. Prior studies have suggested the potential role of substrate accessibility on lipase enantioselectivity (Guieysse et al., 2003).

To investigate further this hypothesis, a novel computational approach, based on motion planning algorithms originally used in robotics to explore constrained high-dimensional spaces (Puech-Guenot et al., 2008), was developed. The use of this approach enabled the computation of accessibility pathways for a set of racemates to reach the buried catalytic site of *B. cepacia* lipase from the protein surface, and the identification of residues potentially hindering their displacement along the active site3.

On the basis of these results, engineering of the narrow active site of BCL has been undertaken to modulate selectively the access of *R* and *S* enantiomers to the enzyme active site. A library of 57 BCL variants was built by iPCR and screened using a high-throughput procedure (Guieysse et al., 2008) to identify active variants towards pNPB hydrolysis. Next, the enantioselectivity of these mutants was evaluated towards a given racemate, the (*R*, *S*) - α - bromo-phenyl ethyl acetate, using a novel screening procedure developed in deep wells. A molecular modelling study of the variants displaying an enhanced enantioselectivity was carried out in parallel. Confrontation of both in silico and experimental results enabled us to better understand molecular determinants controlling BCL enantioselectivity.

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Improving the Activity of Pyranose 2-oxidase via Site Directed Mutagenesis of the Active Site Loop

Dagmar Brugger,[1] Oliver Spadiut,[1] Christina Divne[2] and **Christian Leitner[1]**

1 Departement of Food Sciences and Technology, Division of Food Biotechnology, University of Natural Resources and Applied Life Sciences, Muthgasse 18, 1190 Vienna, Austria, christian.leitner@boku.ac.at

2 Departement of Biotechnology, Royal Institute of Technology, SE-106 91 Stockholm, Sweden

Pyranose oxidase (P2O), a tetrameric flavoenzyme found in wood degrading fungi, catalyzes the C-2 oxidation of several aldopyranoses, including glucose and some monosaccharides commonly found in lignocellulose, to the corresponding 2-keto derivatives. During this oxidation electrons are transferred to oxygen to yield hydrogen peroxide. Pyranose oxidase was regarded as the key biocatalyst in the Cetus process, in which pure crystalline fructose was produced from glucose via the intermediate 2-ketoglucose. Furthermore, some of the 2-keto sugars formed by this enzyme are attractive intermediates in the production of food additives such as tagatose.

Access to the active site is controlled by a flexible loop -DAFSYG- containing three interesting amino acids (Asp, Phe and Thr). Crystal structure of the enzyme substrate complex revealed that this loop is closed over the active site in a complex with the small competitive inhibitor acetate and opened in a complex with the slow substrate 2-flouroglucose. Furthermore Asp542 was found to form a hydrogen bond with the C6 hydroxyl group of glucose and thus favored the oxidation of the C2 OH group. Complete removal of this loop yields an expressable protein although with a 10-fold decreased activity. Saturation mutagenesis was performed of the larger amino acids in the loop region (D452, F454 and Y456) and of H450 at the beginning of the loop. A screening for improved activity with the unnatural substrate galactose was performed, the most active mutants sequenced, purified, crystallized and characterized. The poster will discuss the results we got from the characterization and the crystal structure.

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Structural Implication of T1 Lipase through Rational Design

Thean Chor Leow,[1] Raja Noor Zaliha Raja Abd Rahman,[2] Abu Bakar Salleh,[3] Mahiran Basri,[4] Mohd Basyaruddin Abd Rahman[4]

1 Department of Cell and Molecular Biology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia, adamleow@biotech.upm.edu.my
2 Department of Microbiology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia
3 Department of Biochemistry, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia
4 Department of Chemistry, Faculty of Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia

A thermoalkaliphilic lipase of a novel *Geobacillus zalihae* strain T1 was overexpressed in prokaryotic system. Crystallization of T1 lipase was still possible up to 60 °C. High temperature crystallization is a new discovery in lipase crystallization. The bigger crystal interface grown at 20 °C was solved at 1.5 Å of resolution as due to a balance of hydrophobic interaction, packing rate, and some flexibility. Despite a reduction of one ion pair, networking formed by ion pair and hydrogen bond between loops is stronger than interactions located inside the loop. Denatured protein analysis revealed that mutation D311E gave higher Tm (70.59 °C) as compared to K344R (68.54 °C) and native T1 lipase (68.52 °C).

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Approaching the Function of the C2 Domain in Plant Phospholipases D by Semi-rational Design Approaches

Johanna Mansfeld, Janine Tittel, Sabine Werner, Helge Beer and Renate Ulbrich-Hofmann

Department of biochemistry and Biotechnology, Martin-Luther University, Kurt-Mothes-Straße 3, D-06120 Halle, Germany, mansfeld@biochemtech.uni-halle.de

The phospholipase D superfamily has been recognized to play an important role in many cellular processes, such as signal transduction, membrane trafficking and degradation.[1]

In contrast to microbial phospholipases D (PLD), most plant PLDs are composed of two functional domains – an Nterminal C2 domain which is thought to be involved in the calcium ion-mediated binding of the enzyme to phospholipid membranes via three calcium-binding regions and a C-terminal catalytic domain with the two HKD motifs.

Due to the lack of a three-dimensional model of the plant enzymes, a homology model of the C2 domain of PLD- α from *Brassica oleracea* based on the crystal structures of the highly homologous C2 domains of cytosolic phospholipase A2 and phosphoinositide-specific phospholipase Cδ1 was created. This model was used for the design of various mutants in the C2 domain to get deeper insights into the function of the C2 domain in plant PLDs. Accordingly, a serious of deletion mutants at the N-terminal end of the C2 domain and in the first large loop structure connecting β-sheets 1 and 2 which is involved in calcium ion binding (Ca^{2+}-binding loop 1) was created. In addition, a third ligand for calcium binding was introduced into this loop by exchanging Gly47 by Asp being highly conserved in other C2 domains to enhance the affinity of the enzyme in calcium ion binding. In dependence on the extent of the deletion and the secondary structure element being removed, different effects on activity, structure, and calcium ion binding were observed.

Further insights were gained by exchanging the C2 domains of the highly homologous plant PLDs of the α- type from *B. oleracea* and *Papaver somniferum* which differ considerably in their calcium binding properties.

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P 47

Development of Directed Evolution Assays to Improve the Redox Potential of CotA-laccase

Vânia Brissos,[1] Luciana Pereira,[1] Florentina-Daniela Munteanu,[2*]
Artur Cavaco-Paulo[2] and **Lígia O. Martins**[1]

1 Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Av. Da República, 2780-157 Oeiras, Portugal, lmartins@itqb.unl.pt
2 Departamento de Engenharia Têxtil, Universidade do Minho, 4800-058 Guimarães, Portugal
** Present address: University Aurel Vlaicu of Arad, Department of Chemical and Biological Sciences, Elena Dragoi St, 2, 310330 Arad, Romania*

Laccases, that belongs to the multicopper oxidase family of enzymes, are useful biocatalysts for many and diverse biotechnological applications. Substrate oxidation by laccases involves the Marcus "outer-sphere" mechanism in which the redox potential (E0) difference between the substrate and the T1 Cu site of these enzymes (together with the reorganization energy and the electronic coupling) determines the electron transfer and thus, the enzymatic oxidation rate. Laccases with higher E0 have increased effectiveness and versatility for substrates avoiding the need of redox mediators in many applications. Our studies on the structure-function relationships revealed the limitation of rational approaches to engineer the E0 showing that changes of amino acid residues in direct contact to the metal centre significantly affect the properties of T1 Cu sites of laccases and the enzyme overall reactivity and stability.[1,2] Directed evolution has merged in the past few years as a powerful alternative to rational approaches for engineering biocatalysts. The key to the directed evolution process is the establishment of an efficient expression system and screening system that accommodates the predicted diversity generated by the mutagenesis techniques. In this study the expression levels of cotA were compared in five different *Escherichia coli* host strains, growing in 96 well microtiter plates, under different cultivation conditions. The lower coefficient of variance (15%) was achieved in high-throughput screening assays for standard laccase substrates using crude cell lysates of BL21 and KRX host strains growing under microaerobic conditions. By plotting the decolourisation values of 12 different anthraquinonic and azo dyes by CotA-laccase and the E0 of dyes (measured by cyclic voltammetry) a direct correlation could be observed in accordance with results in the literature. Sensitive and reproducible high-throughput decolourisation screening assays were developed for the identification of high redox variants from evolution libraries. The enzymatic assays developed were tested for the screening of a library (around 2000 clones) from Domain 3 of CotA laccase created by error-prone PCR. The mutagenesis and screening strategies selected allowed for the identification of a variant enzyme with a 2 fold higher activity than the parental strain, therefore suitable for parenting the next generation of random mutagenesis.

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P 48

Rational Design and Directed Evolution of the Maize beta-glucosidase Zm.p60.1

Pavel Mazura

Department of Molecular Biology and Radiobiology, Faculty of Agronomy, Mendel University of Agriculture and Forestry, Zemedelska 1, 61300 Brno, Czech Republic, mazura@sci.muni.cz

The research is focused on investigating modulation of enzyme specificity from the viewpoint of functional relationships between key amino acids that form the entrance to the active site. We are interested in plant β-glucosidases and the maize β-glucosidase Zm-p60.1 is the best described candidate among them. This group of proteins is highly diverse in terms of homologous enzymes and this enables bioinformatics as well as the elucidation of the biological significance of β-glucosidases involved in numerous important processes in plant growing and development.

Rational design in protein engineering has several limitations. Therefore we have used strategy of random site directed mutagenesis followed by directed evolution to investigate the functional relationships between amino acid residues. Generally, this work will shed more light on the complex evolution of enzyme substrate interaction at the active site. Simultaneously, the ability to modulate specificity of β-glucosidases holds considerable promise in terms of biotechnological applications.

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ZymeCAD™, a Versatile and Comprehensive Molecular Simulation Platform. Application in the Study of Antigen-Antibody Complexes

Johannes Mullegger, Paula I. Lario, Tomas Rodinger, Evelyn Mayaan, Ganesh Swami, Powell P.C. Tan, Siddharth K. Srinivasan and Surjit B. Dixit

Zymeworks Inc., 540 - 1385 West 8th Ave., Vancouver, BC, Canada, V6H 3V9, hannes@zymeworks.com

It has become increasingly evident that for a thorough understanding of proteins, it is necessary to account for their dynamic behavior. To base analysis only on the rigid framework as observed in crystal structures is in many cases misleading. Computer simulation methods based on quantum and statistical mechanics as well as empirical data have been developed over many years to appreciate the dynamic behavior of proteins. Zymeworks is developing and validating a comprehensive molecular simulation platform with the goal of using it for rational protein engineering. The available features include a variety of algorithms and their hybrids in the spectrum of deterministic and stochastic molecular simulation methods. These methods provide unique advantages with regard to addressing various aspects of conformational sampling and understanding their impact on biophysical properties.

We shall provide an overview of the functionalities currently available and/or under intensive development within ZymeCAD™. A thorough analysis and comparison of molecular dynamics simulation results acquired with the ZymeCAD™ platform with those obtained from an established academic molecular dynamics package (NAMD) has been undertaken for validation purposes. Our validation studies are based on anti-fluorescein antibody 4-4-20 and its mutant 4M5.3 which has 1800 fold improved affinity over the wild type. The 4-4-20 is a ~30 kDa protein known as the single chain Fv which comprises of the light and heavy chains of the complementarity determining regions. This constitutes the antigen binding domains in the intact immunoglobulin, which is 150 kDa in size. The antibodies have previously been extensively characterized in the research group of D. Wittrup through structural, thermodynamic, kinetic and mutational methods.

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P 50

In vitro Reactivation of Inclusion Bodies and Homologous Expression of Recombinant Glutamate Dehydrogenases from *Halobacterium salinarum*

Nayla Munawar and Paul C. Engel

School of Biomolecular and Biomedical Sciences, Conway Institute, Belfield, University College Dublin, Dublin 4, Ireland, nayla.munawar@ucd.ie

The folding, stability and therefore, the function of halophilic proteins depend critically on salt concentration, thus the enzymes from halophiles may serve as model proteins for biotechnological applications, such as biotransformation whereas a low water environment is required. *Halobacterium salinarum* is one of the halophilic archaea that require more than 3M NaCl for growth. The enzymes of this organism have thus been adapted to be active and stable in hypersaline conditions, making them strong candidates as robust industrial enzymes. Ingoldsby et al.[1] reported four glutamate dehydrogenase genes (GDH A, A2, B and X) after amplifying the genomic DNA of colourless strain NRC-36014 of *Halobacterium salinarum*. To compare their properties heterologous or homologous gene expression is essential.

Most typical enzymes from halophilic archaea require high concentrations of salt for activity and stability. Expressed in *E.coli*, these enzymes are inactive unless refolded in the presence of salt in-vitro. The over-expression of the four recombinant GDH(s) in *E.coli* produced inclusion bodies, and in-vitro refolding protocols explored so far have yielded only insoluble aggregates or soluble inactive protein. Owing to this problem we need a homologous expression system to keep these proteins in their active native state. *Haloferax volcanii* is an obligate halophile. It is a genetically stable prototroph that has become a model organism for molecular genetic studies of the archaea. The presence in Haloferax volcanii of an efficient transformation system, several shuttle vectors and selectable markers has made a wide variety of molecular genetic studies possible. NAD^+-specific GDHX from *Halobacterium salinarum* has been cloned successfully in pRV1-tna plasmid having a tryptophan-inducible promoter. Transformation was performed by the polyethylene glycol (PEG) method and transformants were selected on 18% modified growth medium (MGM) with novobiocin as a selective marker. *Haloferax volcanii* with the pRV1-tna GDHX plasmid and induced by 4 mM tryptophan has 50 times more NAD^+-specific GDH activity than cells without the plasmid.

Our current efforts have therefore been focused to improve expression levels using different concentrations of tryptophan. Furthermore, attempts to purify the over-expressed protein by column chromatography will be reported.[2]

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P 51

Codex™ Diagnostic Panels: Using Tunable Biocatalysts to Accelerate Process Development

Vesna Mitchell, Christopher Savile, Anke Krebber, Vesna Gavrilovic, Erika Segraves, Galit Meshulam-Simon, Sarena Tam and Gjalt Huisman

Codexis Inc., 200 Penobscot Drive, 94063 Redwood City, California, USA, vesna.mitchell@codexis.com

A Codex™ diagnostic enzyme panel is a set of functionally diverse biocatalyst variants that are pre-tuned to accept a wide range of substrates, produce different stereoisomers, be chemically robust (e.g., solvent and thermal stable), and be manufactured at commercial scale. The plate variants are also designed to yield structure-function information that can be used to provide additional diversity. For example, screening of a pharmaceutically relevant ketone with commercially available ketoreductases showed no enzyme with desired activity. However, screening of the ketoreductase Codex™ diagnostic panel plate identified multiple variants with a range of activities and enantioselectivities. Based on the sequence-activity data from this panel, a new plate of customized variants was designed that showed increased activity and enantioselectivity toward the desired enantiomer. This approach allows for quick determination of the feasibility of a biocatalytic route and provides a diagnostic tool for further optimization using directed evolution.

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Kinetic Studies of Phenylalanine Dehydrogenase from *Bacillus sphaericus* and Derived Mutants with a Variety of Substrates

Rajesh Narayanan and Paul C. Engel

Department of Biochemistry, School of Biomolecular and Biomedical Science, Conway Institute, University College Dublin, Belfield, Dublin 4, Ireland, rajesh.narayanan@ucdconnect.ie

Our group is developing novel biocatalysts based on phenylalanine dehydrogenase by mutagenic alteration. In order to use a knowledge based site-directed mutagenesis approach, it is important to understand the way the enzyme works, including the order of substrate addition. To this end, detailed kinetic studies have been carried out on wild type phenylalanine dehydrogenase from *Bacillus sphaericus* and on mutants in which substrate specificity has been altered by site-directed mutagenesis. Full initial-rate equations have been extracted for both the wild type and mutant enzymes by systematically varying the concentrations of both the coenzyme, NAD^+, and the substrate, phenylalanine, and doing the same for analogues. The kinetic constants have been used to apply mechanistic tests to find out the order of substrate binding. From the initial experiments, we were able to rule out some of the candidate mechanisms to explain the order of substrate binding. The coenzyme analogue deamino NAD+ was prepared chemically from NAD^+ with nitrous acid and purified chromatographically to use for the experiments. Initial results suggested that the enzyme follows a random-order mechanism for this two-substrate reaction. On the basis of this mechanism, the dissociation constants of all the binary and ternary enzyme substrate complexes can be calculated from the parameters obtained through the initial experiments.

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An Upside-down Substrate Flip within the Active Site of Evolutionary Related Beta and Alpha Glycopyranoside Hydrolases Explains their Mechanistic Relationship

Wim Nerinckx

Department of Biochemistry, Physiology and Microbiology, Unit for Molecular Glycobiology, Ghent University, K.L. Ledeganckstraat 35, 6th floor, B-9000 Ghent, Belgium, wimnerinckx@lavabit.com

Within the research area of glycoside hydrolases and their catalytic mechanisms, two pairs of distant but clear structural and evolutionary relationships have recently been discovered between enzyme families that are acting on beta and on alpha glycopyranosidic bonds.[1]

a) Enzymes from families 1, 35 and 42 belonging to GH-clan A are retaining hydrolases acting on beta glycopyranosides, but are found to be related to GH-family 14 that contains alpha-inverting enzymes; all of these are TIM-barrel folded.

b) Enzymes from family 15 belonging to GH-clan L are inverting hydrolases acting on alpha glycopyranosides, but are found to be related to GH-family 94 that contains beta-inverting enzymes; all of these are (α/α)6 folded.

This is remarkable since one expects that evolutionary related enzymes should have a relatively similar set-up of their respective catalytic machinery, and thus should be catalyzing their respective reactions with an overall reaction itinerary that is relatively similar. On closer inspection of crystal structures from these enzymes (this work), several extra correlations are observed within both sets of evolutionary relationships:

a) In the fist set, the beta-acting families 1, 35 and 42 contain anti protonators (proton donor near-lateral to the glycosidic ring and close to C2-OH) whereas the related alpha-acting family 14 contains syn protonators (proton donor near-lateral to the glycosidic ring and close to O5).[2,3]

b) The second set shows the inverse correlation, i.e. the alpha-acting family 15 contains anti protonators whereas the related beta-acting family 94 contains syn protonators.

c) In those liganded complexes that are –1/+1 subsite-spanning –and this is observed for all GH-enzymes– the scissile bond is in a conformation where the exo-anomeric effect is present when the enzyme is an anti protonator, whereas it is by about 120 degrees out of the exo-anomeric effect when the enzyme is a syn protonator.

d) Overlaps of liganded crystal structures of the related beta and alpha hydrolyzing enzymes show for both sets that the respective active site machineries are still quite similar with the catalytically essential residues at roughly the same place, but that the respective glycopyranoside moieties within the crucial –1 subsite are flipped by 180 degrees. This directly explains the observed beta-anti with alpha-syn (and vice versa: alpha-anti with beta-syn) correlations.

All of this is in perfect agreement with the respective ALPH-compliant classical substitution itineraries for beta and alpha O-glycopyranosidic bonds that are observed to invariably occur within glycopyranoside hydrolases.[4] Indeed, the forward Deslongchamps itinerary for betas (4C1 ground state → 1S3 pre-TS → 4H3 TS → 4C1 end state) is, when the sugar is turned upside-down, in its overall atomic movements highly similar to the forward alternative-ALPH itinerary for alphas (4C1 ground state → 3S1 pre-TS → 3H4 TS → 1C4 initial end state), providing a simple explanation for how the evolutionary related GH-enzymes are mechanistically related. Moreover, a beta 1S3 pre-TS with the glycosidic bond in the exo-anomeric effect is very similar in shape to an alpha 3S1 pre-TS that has this bond by 120 degrees out of the exo-anomeric effect; idem but vice versa with a beta 1S3 pre-TS out of the exo-anomeric effect compared to an alpha 3S1 pre-TS in its exo-anomeric effect. Since syn protonators quite probably have evolved from ancestors that originally were anti protonators acting on glycosidic substrates where the ground-state stabilizing exo-anomeric effect is present,3 the above then suggests how this could have happened, i.e. initially not by adaptation of the enzyme but by a "lucky" fit of a substrate with inverted anomeric configuration, entering the –1 subsite upside-down and with the glycosidic bond out of the exo-anomeric effect.

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EnBase™ - High Density Cell Cultivation for High Throughput Screening

Antje Neubaeur,[1] Maria Ruottinen,[1] Antti Vasala,[1] Johanna Panula-Perälä,[2] Russell Golson[1] and Peter Neubauer[1,2]

1 BioSilta Oy, Pentti Kaiteran Katu 1, P.O.Box 4300, 90014 Oulu, www.biosilta.com, polarcircle@gmx.de
2 Bioprocess Engineering Laboratory, Department of Process and Environmental Engineering and Biocenter Oulu, University of Oulu, Oulu, Finland

EnBase™ is an advanced cell culture technology which enhances cell growth, 20 to 50 times higher densities can be achieved when compared with standard shaken cultures. The EnBase™ technology is based on the industrial fed-batch strategy, but it uses an internal substrate delivery system instead of an external supply of the substrate through pumped systems. EnBase™ is applicable to automated and manual systems and is especially advantageous for high throughput crystallisation, protein expression, and genome sequencing approaches where multiwell plates are used as well as in shake flasks, e.g. for protein labelisation. EnBase™ controls the cell growth in a way that anaerobiosis and overflow metabolism, which are generally connected to batch cultures, are circumvented. BioSilta (www.biosilta.com) has developed an EnBase™ product platform for different scales of cultivation. The kits are ready to use and include the delivery platform and the cultivation medium. EnBase™ 96 and 48-DWP are a ready-made micro-well kits which need only to be inoculated with a mixture of the delivered medium and the starter culture for growth to commence. EnBase™ in shake flask format can be used for high cell density shake flask cultivation for the production of much greater amounts of the product, eventually replacing laborious and expensive fermented cultivations. In development is the EnBase™ Falcon tube system, which bridges the microwell and shake flask scales.

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TIM-based Biocatalyst Platform for Chiral Synthesis of Chemical Compounds

Peter Neubauer, Marco Casteleijn, Mirja Krause, Mikko Salin, Markus Alahuhta, Matti Vaismaa, Nanna Alho, Sampo Mattila, Marja Lajunen and Rik Wierenga

Technical University of Berlin, Straße des 17. Juni 135, 10623 Berlin, Germany, peter.neubauer@oulu.fi

Our aim is to exploit the enzyme triosephosphate isomerase (TIM) as a scaffold to create artificial enzymes with a wide substrate range. Therefore we apply structure-based mutagenesis in a combination of rational single point directed mutagenesis and randomizing strategies. Here we demonstrate the feasibility of the approach by generating a new monomeric arabinose isomerase.

Additionally we present a new technology (EnBase™)for high cell density cultivation of recombinant *Escherichia coli* in microwell plates which enables screening of mutant libraries by reaching >10x higher volumetric yields for target proteins.

Triosephosphate isomerase (TIM) is a glycolytic enzyme with very high substrate specificity which catalyzes the interconversion of dihydroxyacetone phosphate (DHAP) and D-glyceraldehydes-3-phosphate (D-GAP). The wild type enzyme is a dimer, and each subunit has the classical TIM-barrel fold. Several loop modifications resulted in a monomeric enzyme (ml1TIM) which remains active, although less than the wild-type. Subsequently this has resulted in a new variant (A-TIM) with an extended binding pocket. A-TIM and some site directed variants of A-TIM were the starting points for the creation of a new arabinose isomerase.

The substrate binding loops of A-TIM were modified by site-directed mutagenesis to obtain different mutant enzymes as starting points for directed evolution. In former studies it has been shown how important a single point mutation for the generation of a competent active site is. Directed evolution was performed in a fully random approach (epPCR) and a targeted approach (TA). The extensive structural knowledge of TIM has been the basis for the choice of amino acids that were targeted through Megaprimer PCR (TA) for random mutagenesis. Different libraries were the starting point for the directed evolution approach, which yielded new arabinose isomerases, which were selected by screening for complementing an arabinose isomerase knockout mutant of *E. coli*.

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Protein Design for New Activities Through Catalytic Loop Exchange in the (βα)8-fold

Ochoa-Leyva Adrian, Saab-Rincón G., Sánchez-López F. and Xavier Soberón

Departamento de Ingeniería Celular y Biocatalisis, Instituto de Biotecnología, Universidad Nacional Autonoma de Mexico, Mexico City, Mexico, aol@ibt.unam.mx

Natural proteins optimized through evolution, represent potentially attractive starting materials for the creation of novel activities. Combining protein design and directed evolution with scaffold-based protein libraries provides an excellent route to engineer new functions. The advantage of such libraries depends on how tolerant the scaffolds are to randomization, because the selected variants must remain folded and soluble. The (βα)8 barrel scaffold, due to its catalytic and structural versatility, is an ideal starting point for the engineering of novel enzymatic activities by protein design and directed evolution.

Herein is described a new strategy to exchange loops on a (βα)8-fold. Phosphoribosyl anthranilate isomerase (TrpF) was chosen as scaffold and the tolerance of TrpF to loop exchange was investigated by fusing the libraries to the chloramphenicol acetyl transferase (CAT) coding gene, as in vivo folding reporter. The interexchange was directed towards 3 of the 8 (βα) loops of the TrpF structure and 13 different (βα) loops belonging to 8 distinct (βα)8 enzymes with diverse functions were used in those positions. To warrant the best structural solution, saturation mutagenesis at the last 2 amino acid positions pointing towards the inside of the barrel, located within the α β-strands and preceding the exchanged loop, was adopted.

Our results showed that 25% to 75% of the generated mutants in the different libraries were folded. Some variants were selected in absence of CAT, and their stability was studied by means of circular dichroism. This highlighted the overlapping of the far UV-CD spectra of both the chimeric proteins and the TrpF WT enzymes, even if subtle changes were noticed, suggesting some structural changes. However, all spectra demonstrated features of folded proteins. Additionally, the introduction of variability at the “hinges” connecting the loops with the scaffold also exhibited a noticeable effect for obtaining folded proteins. Moreover, it is also important to emphasize that each position accepted foreign loops of different sizes and sequences, The proportion of chimeric proteins that remained foldable after the introduction of foreign loops also indicates that our approach to afford useful libraries for subsequent functional screening, is successful.

Therefore, the design of a general method to exchange variable-sized loops within (βα)8-fold, in an efficient enough way to provide a reasonable starting point for the selection of new activities, was proven. In conclusion, one could speculate that even modest diversion from an original activity and specificity would benefit from the employment of loops as modular components of variability.

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New Nitrile Hydratases for Biocatalysis

Linda G. Otten, Sander van Pelt and Isabel Arends

Biocatalysis & Organic Chemistry, Delft University of Technology, Julianalaan 136, 2628 BL Delft, The Netherlands, l.g.Otten@tudelft.nl

Nitrile hydratases (NHases, E.C. 4.2.1.84) were discovered 25 years ago in studies of microbial degradation of toxic cyano-group-containing compounds.[1] They can catalyse the transformation of nitriles into the corresponding amides. The use of NHase in synthetic chemistry is especially interesting in conversions where the substrate or the product is labile at the high temperature or extreme pH employed in chemical nitrile hydrolysis. Besides the mild reaction conditions, the potential advantage of using an NHase as catalyst is a high chemo-, regio- and enantioselectivity.[2] However, also in the production of bulk chemicals like acrylamide NHases offer a green alternative to chemical nitrile hydrolysis.

Enantioselectivity is an important aim in (bio)catalysis. Unfortunately not many enantioselective NHases have been described yet. In order to expand the availability of NHases, we enriched samples from soda soils and lakes on different nitriles and found a few organisms containing NHase activity.[3] The aim of our research is to study these NHases and use them as a biocatalyst. Therefore we are working on an expression system, which will allow us to produce and purify the enzyme. In order to pick the gene from these new and unknown organisms, we designed degenerate primers based on known NHases. The identification and isolation of these genes will be described.

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Rational Design of Restriction Endonuclease Specificity through Computational Analysis and Subunit Rearrangement

Sebastian D. Pawlak[1,2] and Janusz M. Bujnicki[1]

1 Laboratory of Bioinformatics and Protein Engineering, International Institute of Molecular and Cell Biology, Trojdena 4, 02-109 Warsaw, Poland, sebastian@genesilico.pl
2 Institute of Biochemistry and Biophysics,Polish Academy of Science, Warsaw, Poland

Restriction Endonucleases (REases) are known for precise recognition, binding and hydrolyzing phosphodiester bonds inside or in close proximity of the target sequences. These features make them model molecules for studying the structural and biochemical mechanisms of specific protein-DNA interactions. They are also irreplaceable tools in molecular biology as they act as specific scissors for DNA recombination, molecular diagnostics and many other applications. The number of known REases (3800 in 2008) doesn't reflect the number of unique recognition sites (260). as most of them are isoschisomers that recognize identical sequences. Therefore there is strong interest in engineering existing enzymes to obtain novel "unnatural" specificities.

Here we describe a novel approach for changing the specificity and function that was applied for Bsp6I REase that has an unusual cleavage pattern GC'NGC.

In our previous work we generated a computational model of Bsp6I structure and verified it experimentally. Based on the model we suggested that mutations in the region spanning amino acids 91-94 could generate new contacts with the unspecified middle base pair and narrow the existing specificity. To introduce asymmetry into dimeric enzyme that would reflect asymmetry of pentanucleotide recognition site, we have constructed the heterodimeric enzyme with one subunit containing mutations in position 94 (E94R or E94K) while the other subunit remained wild-type. In comparison to the wt enzyme such Bsp6I variants possessed 10 fold lower activity toward GCSGC sequence (where S =G or C) retaining unchaged activity against GCWGC (where W=A or T).

To avoid the possibility of dissociation of heterodimeric forms and reconstitution of homodimers, we have covalently attached two wt enzyme subunits with short GSGGG linker and created single chain (sc) Bsp6I variant with activity and specificity similar to homodimeric form. We had also constructed sc enzyme where one subunit remained wildtype while the second carried mutation E94K. It showed lowered activity but sequence specificity was similar to the heterodimeric form. We also fused the wild-type subunit with mutant E76A that is known to be catalytically inactive. This scBsp6I variant showed sequential cleavage of each DNA strand with preference for initial cleaving of the strand with T in the central position of the recognized sequence.

Our results indicate that combining computational protein structure modeling with different protein engineering methods could be a promising strategy for modifying properties of REases such as sequence specificity.

P 59

Directed Evolution of New Galactose Oxidase Variants for Glycan Labelling

Julie Rannes,[1] Franck Escalettes,[1] Avgousta Ioannou,[1] Carsten Behrens,[2] Sabine Flitsch[1] and Nicholas Turner[1]

1 Manchester Interdisciplinary Biocenter, School of Chemistry, University of Manchester, 131 Princess Street, Manchester, M1 7DN, UK, julie.rannes@manchester.ac.uk
2 Novo Nordisk A/S, Novo Nordisk Park, 2760 Måløv, DK

The labelling of proteins using bio-orthogonal chemical reporters represents a feasible method for site-specific modification of proteins.[1] The strategy involves the insertion of unique functionality into the target of interest followed by chemical labelling with a small molecule probe. A commonly used bio-orthogonal chemical reporter involves aldehyde groups that can be tagged with hydrazide derivatives under mild conditions.[1]

The enzyme galactose oxidase is a copper containing enzyme that oxidises the C-6 hydroxyl group of galactose to the aldehyde which can be selectively modified for glycan labelling applications.[2] However, the wild-type enzyme is highly specific towards galactose.[3] The aim of this project is to employ directed evolution approaches to identify variants of galactose oxidase that can oxidise the 6'-OH group of other sugars found in glycoproteins. Such an approach will allow the insertion of a bio-orthogonal aldehyde functionality into expressed glycoproteins for sitespecific glycan labelling. Optimally, these galactose oxidase variants should have good activity to ensure their usefulness for glycan labelling.

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P 60

Tailoring Alpha-transglucosidase Specificity for Programmed Chemo-enzymatic Synthesis of Complex Bacterial Oligosaccharides

Remaud-Simeon Magali

Laboratoire Ingénierie des Systèmes Biologiques et des Procédés, Institut National des Sciences Appliquées, University de Toulouse, 135, Avenue de Rangueil, 31077 Toulouse, France, remaud@insa-toulouse.fr

Focused on the synthesis of serotype-specific *Shigella flexneri* oligosaccharide haptens for third generation vaccines against shigellosis, our approach was based on the exploitation of enzyme engineering to circumvent sugar organic synthesis boundaries and create new glycoenzymes designed on purpose to participate in a programmed chemo-enzymatic pathway.

Amylosucrase, an alpha-transglucosidase of glycoside-hydrolase family 13, naturally transfers glucosyl residues from sucrose donor to glucose acceptor to synthesize an amylose-like polymer1. Molecular modeling of this enzyme, in complex with non natural acceptors (L-rhamnose and N-acetyl-glucosamine derivatives) was first used to guide the semi-rational construction of a small size mutant library directed to the acceptor binding site. The aim was the design of new amylosucrase mutants specific for the glucosylation of the desired protected acceptors and the synthesis of intermediates conveniently protected for subsequent chemical elongation. The challenge was double: new stereo and regio-specificities had to be created and specificity toward sucrose donor had to be maintained. Library screening enabled the isolation of two mutants showing new and/or tremendously enhanced specificities toward the non-natural protected sugar acceptors. The glucosylation products were successfully used at for the synthesis of the O-antigen motifs of *S. flexneri* serotype 3a and 1b. Our work illustrates the potential of protein engineering to create on purpose new glucosylation tool and develop original chemo-enzymatic pathway for glycochemistry.

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P 61

Fused Cytochrome P450 Enzymes for more Effective Biohydroxylations

Aelig Robin

Manchester Interdisciplinary Biocentre, Manchester University, 131 Princess Street, M1 7DN Manchester, UK, aelig.robin@manchester.ac.uk

P450 monooxygenases catalyze important and chemically difficult reactions under mild conditions in a regio- and stereoselective manner. The majority of these enzymes only work as part of a multiprotein complex with redox partners providing electrons from NADH or NADPH cofactors to the heme containing subunit. The cofactor requirement is therefore a major drawback of the oxygenases for the biotechnology sector.

Our group has recently discovered a new class of fused P450 oxygenases (P450RhF, from *Rhodococcus* sp strain NCIMB 9784) in which the reducing equivalents are providing by a novel reductase in a fused arrangement. We have generated a novel fused protein by linking the reductase domain of P450RhF to the heme unit of a different P450 monooxygenase (P450 camphor). Chimeric P450 enzymes (differing by the length of the linker) were expressed in *E. coli* and used in whole cell biotransformations for the conversion of camphor to 5-exo-hydroxyl camphor. The results show that P450RhF reductase domain provides a scaffold for generating self-sufficient P450 enzymes.

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A Panel of Cytochrome P450 BM3 Variants To Produce Drug Metabolites and Diversify Lead Compounds

Andrew M. Sawayama, Michael M. Y. Chen
and Frances H. Arnold

Department of Chemistry and Chemical Engineering, California Institute of Technology, 1200 E California Blvd, 91125 Pasadena, CA, USA, sawayama@cheme.caltech.edu

The cytochrome P450 is a proven design for the engineering of C-H activation catalysts. Here, we demonstrate that variants of a soluble bacterial P450 (P450BM3 from *Bacillus megaterium*) can act as general C-H activation catalysts by mediating regioselective hydroxylation of complex drug-like structures. A small panel of variants covers the breadth of reactivity of human P450s by producing 12 of 13 natural metabolites for three different drugs. We also demonstrate that highly active P450 BM3 enables preparation of the individual metabolites at clinically relevant scales. Emphasizing the generality of this P450 C-H activation scaffold, the engineered variants produce novel metabolites by catalyzing reactions at carbon centers beyond those targeted by human P450s. To improve utility, we explore a basis for predicting the reactivity of this enzyme panel on as yet untested substrates.

P 63

Isothermal DNA Amplification in Microdroplets

Yolanda Schaerli,[1,2] Viktor Stein,[1] Michelle M. Spiering,[3] Stephen J. Benkovic,[3] Chris Abell[2] and Florian Hollfelder[1]

1 Department of Biochemistry, University of Cambridge, 80 Tennis Court Road, Sanger Building, CB2 1GA Cambridge, UK, ys316@cam.ac.uk
2 Department of Chemistry, University Of Cambridge, Cambridge, UK
3 Department of Chemistry, Pennsylvania State University, University Park, PA 16802, USA

In vitro compartmentalisation is an approach that employs water-in-oil droplets as microreactors, which can be used to link genotype to phenotype in directed evolution experiments. Each droplet contains a gene and an in vitro transcription and translation (IVTT) system that contains all the necessary ingredients for protein expression and detection of optically active product is used to sort the droplets containing clones that successfully catalyse a reaction. Microfluidic systems can be used to generate and manipulate such microdroplets in a more controlled manner than in bulk emulsion. The microdroplets produced in a microfluidic device are highly monodisperse and can be fused, split, incubated and sorted.

For a successful evolutionary process it is important that initially weak activities can be detected in the initial rounds of directed evolution, to avoid that no clones can be recovered or to maintain diversity after selection. The detection of small amounts of product produced by mutants with low activity is a challenge, because in 'monoclonal' droplets (= one gene per droplet) only a limited amount of protein and consequently product is produced that may be below the detection limit. The amount of protein produced with IVTT depends on the amount of template DNA, so DNA amplification in microdroplets would be strategy to extend the dynamic range of such directed evolution experiments.

For this purpose we established an isothermal DNA amplification method that amplifies circular double-stranded template DNA to result in a defined linear double -stranded product by a rolling circle mechanism. The amplification system is based on the T4 replication machinery, which consists of the exonuclease-deficient DNA polymerase (gp43), the helicase (gp41), the helicase loading protein (gp59), the clamp protein (gp45), the clamp loader proteins (gp44/62) and the single-stranded DNA-binding protein (gp32). The reaction is performed at 37 °C and ca n be used to amplify whole plasmids (>3000 bp) around 3000 times in less than an hour. In contrast to the system used by Xu and Co-workers[1] we include a nicking endonuclease in the amplification reaction, which prevents the production of high-molecular weight concatomers and therefore increases amplification power. Current work includes the integration of this isothermal DNA amplification method with IVTT in microdroplets and application on model selection using the covalent SNAP-tag mediated genotype-phenotype linkage developed by Stein et al.[2] The utility of our DNA amplification strategy is benchmarked by measurement of enrichment and recovery efficiency.

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P 64

Carba Analogues of PAF - Enzyme Assisted Syntheses and Biological Activities

B. Jakob, K. Lange and **Manfred P. Schneider**

Department Organische Chemie, FB C, Bergische Universität Wuppertal, Gaussstrasse 20, 42097 Wuppertal, Germany, schneid@uni-wuppertal.de

Platelet activating factor (PAF), an ether lipid, is interacting with the G-protein coupled PAF receptor and thus activates multiple intracellular signalling pathways.[1] It is an important mediator of platelet aggregation, inflammation and anaphylaxis. Structural analogues of PAF such as 1-Octadecyl-2-O-Methyl-sn-glycero-3-phosphocholine (ET-18-O-CH3) display anticancer properties.[2] Since PAF is metabolically instable with a half-life of only a few minutes due to hydrolysis of the acetate function in the sn-2 position by acetyl hydrolases, numerous derivatives such as alkyl ethers, thio ethers and carbamoyl derivatives have been prepared and studied regarding their biological activities. All of them, however, change the stereo electronic character of PAF and we thus decided to synthesize a series of carba- analogues of these molecules in which the sp3 oxygens of the acetyl functions are replaced by sp3- carbons. This change results in isosteric mimics of the natural molecules with minimal deviations regarding bond angles and bond distances.[3] Key steps are enantioselective, enzyme (lipase) catalyzed esterifications and hydrolyses, thereby differentiating between either the enantiotopic hydroxy groups in achiral 2- substituted 1,3 diol precursors or the enantiomers of the corresponding esters.[4] Both enantiomeric series of the title compounds were synthesized, carrying a variety of alkyl and acyl groups with the potential of also varying the polar head groups.[5] The thus obtained molecules are currently tested regarding their biological activities. While the originally expected antitumor and antiproliferative activities are only weak to moderate, first results indicate that the title compounds (somewhat surprisingly) display extremely high antimicrobial (antimycotic) activities towards micro organisms such *Mycobacterium vaccae*, *Candida albicans* and *Pennicillium notatum* comparable to commercial drugs such as ciprofloxacin and amphotericin B.

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P 65

Changing the Substrate Scope of Transaldolase B *E. coli* towards Non-phosphorylated Compounds

Sarah Schneider, Georg A. Sprenger
and Anne K. Samland

Institute of Microbiology, Universität Stuttgart, Allmandring 31, 70569 Stuttgart, Germany, mbsch@imb.uni-stuttgart.de

The application of dihydroxyacetone phosphate (DHAP)-dependent aldolases in biocatalysis is limited by their strict donor specificity for DHAP. Besides different approaches to facilitate synthesis of DHAP,[1] efforts are made to find or evolve aldolases which are able to use the non-phosphorylated analog dihydroxyacetone (DHA) as substrate.[2] In our group we are investigating two enzymes, fructose 6-phosphate aldolases (FSA) and transaldolase B (TalB), which are able to use DHA either directly in an aldolase reaction or indirectly in a transaldolase reaction. Both enzymes are highly similar with respect to their 3D structure, primary sequence and mechanism.[3-6] TalB catalyzes the transfer of a DHA moiety from a donor (D-fructose 6-phosphate, F6P) onto an acceptor (D-erythrose 4- phosphate, E4P). FSA forms F6P from DHA and D-glyceraldehyde 3-phosphate (GAP). It does not catalyze the transaldolase reaction. A structure-based sequence alignment revealed differences in amino acid sequence close to the active site which might be responsible for the change in activity. Hence, we generated site-saturation mutagenesis libraries at 11 positions in the active site of TalB. The libraries were screened for the formation of F6P from DHA and GAP and with an enzyme-coupled color assay in which lilac formazane was formed by reduction of p- Nitro-blue tetrazolium chloride.

In the screening of the 11 libraries we found 23 positive clones in the F178X-Library all of which exhibit a tyrosine instead of a phenylalanine residue at position 178. TalBF178Y is able to catalyze the transfer of DHA from F6P onto E4P (14U/mg) and is able to use DHA as a substrate to form F6P (7U/mg).[7] The single amino acid exchange in TalBF178Y increased the activity for the formation of F6P considerably (more than 70-fold compared to wildtype). The X-ray structure of TalBF178Y was solved at a resolution of 1.4 Å (Sandalova & Schneider, Karolinska Institutet, Stockholm, Sweden). A sulphate residue was found in the active site near the residues Arg181, Ser226 and Arg228. This suggested that these amino acid residues are responsible for the binding of the phosphate group of the acceptor. Based on this assumption we generated in a second round of mutagenesis three site-saturation mutagenesis libraries. These libraries were screened for the formation of D-fructose with a modified form of the enzyme-coupled color assay described above. The libraries where screened for a lower Km for D,L-glyceraldehyde than the TalBF178Y (more than 80mM) with 10mM and 100mM D,L-glyceraldehyde as lower and upper limit, respectively. In the Phe178Tyr/Arg181X-Library we found one mutant variant (amino acid replacement from Arg to Glu) which exhibited a Km for D,L-glyceraldehyde of 20mM compared to 2mM for GAP of the parent (TalBF178Y). In two rounds of site-saturation mutagenesis we changed TalB of *E.coli* from a transferase towards a mixed lyasetransferase form and additionally lowered the Km for a non-phosphorylated acceptor like D,L-glyceraldehyde from more than 80mM to 20mM. Further studies will investigate to which extend the TalBF178YR181E mutein is able to utilize different non-natural and non-phosphorylated compounds beside D-glyceraldehyde.

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Engineering the Enolase Active Site Pocket: Is the Second Magnesium Ion Essential for Enolase Activity?

Bettina Schreier and Birte Hoecker

MPI for Developmental Biology, Max Planck Society, Spemannstr. 35, 72076 Tuebingen, Germany, bettina.schreier@tuebingen.mpg.de

Enolase, a central enzyme in glycolysis, is the most prominent member of the enolase superfamily. All members consist of a central (αβ)8-barrel domain containing the catalytic residues plus an a+b capping domain that influences substrate specificity. They share a common initial reaction step: The abstraction of the alpha-proton of a carboxylate substrate by a general base. The resulting enolic intermediate is stabilized by a conserved active site Mg^{2+}.

Enolase itself is the only member, in which the intermediate is coordinated by a second Mg^{2+} (MgII). MgII interacts with one carboxylate oxygen (O1) and a phosphate group oxygen of the substrate 2-phosphoglycerate (2-PGA). A conserved serine (S39 in yeast enolase1) of the capping domain is the only residue that interacts directly with MgII. Two water molecules positioned by an aspartate (D321) from the barrel domain complete the coordination sphere of MgII.

No appreciable shift in the position of the substrate skeleton upon the transition from substrate to product was reported. Thus, in addition to stabilization of the intermediate, the positive charge of MgII might hold the carbon skeleton of 2-PGA in a conformation that facilitates the formation of a double bond in the intermediate.

We want to investigate whether MgII can be replaced by positively charged side-chains that provide an alternative means to stabilize 2-PGA in the enolase reaction. To test our hypothesis yeast enolase1 point mutants were created by a rational design approach. In all mutants, the MgII coordinating residues, S39 and D321 were replaced to remove all direct and indirect protein-MgII interactions. Additionally, positively charged side-chains were introduced into the active site pocket to position the charged atoms in close proximity to 2-PGA's carboxylic oxygen O1.

All mutants appear to be correctly folded judged by far-UV CD and fluorescence spectroscopy. The mutants exhibit measurable enolase activity in two independent assay systems. While we observe only small shifts in KM, the turnover appears greatly reduced compared to the wildtype enzyme.

In the control mutant, only the MgII coordinating residues S39 and D321 were replaced, without providing an alternative positive charge for stabilization of the intermediate. This variant, that supposedly can no longer bind MgII, exhibits no measurable enolase activity in both assay systems.

We conclude that although MgII is essential for enolase activity, it can be partly restored by the introduction of an alternative positive charge. To further prove our hypothesis and test our structural predictions, we are now working on the crystallization of enolase mutants in complex with 2-PGA and Mg^{2+}.

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Directed Evolution and Specific Engineering of Industrial Biocatalysts for Enantioselective C-C-bond Formation

Martin Schürmann, Oliver May and Daniel Mink

DSM Pharmaceutical Products / Innovative Synthesis & Catalysis, P.O. Box 18, NL-6160 MD Geleen, The Netherlands, martin.schurmann@dsm.com

Formation of chiral C-C bonds in a stereo controlled fashion is a challenge in synthetic chemistry. Enzymes that catalyze C-C bond formation however are typically enantioselective and/or diastereoselective. We have developed a number of aldolases and hydroxynitrile lyases biocatalyst platforms that are suited for C-C bond formation and have carried out directed evolution and site directed mutagenesis to make these enzymes more suited for industrial use in the production of high-value chiral building blocks for pharma, flavour and fragrance, and fine chemical industry.

This presentation will focus on the design of 2-Deoxy-D-ribose aldolase (DERA) which is a very versatile aldolase of industrial use. This enzyme has been shown in a pioneering work by Wong and co-workers to carry out subsequent (tandem) aldol condensations using cheap starting materials to provide access to multichiral compounds in a single synthesis step. One product class of particular interest are statin intermediates which can be produced using DERA. However, high enzyme consumption (g DERA / g product) is limiting the industrial use of this enzyme.

We have carried out directed evolution, saturation mutagenesis and site specific changes to obtain variants of DERA that show significantly improved performance for production of statin intermediates under industrial relevant conditions.

The path from the first transformations with wild type enzymes in the laboratory, via the design and improvement of enzyme mutants, their production and application on pilot and commercial scale as well as novel applications will be discussed.

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New Cage Compounds as Potential Influenza A Inhibitors: Synthesis and Docking/QSAR study

Tatyana Shubina

Computer-Chemistry-Center, Nägelsbachstraße 25, 91052 Erlangen, Germany, tatyana.shubina@chemie.uni-erlangen.de

The influenza A virus, with 27 known subtypes that range from low to high pathogenicity in the bird population, also infects humans and other mammals. Subtype H5N1, currently known as avian or bird flu, is of particular interest because of its increasing pathogenicity and its ability to form a new viral subtype to which there is no native immunity in human hosts.

Adamantane derivatives have been used successfully for the prevention and treatment of influenza A virus infection for more than 30 years. It is proposed that these drugs inhibit influenza A virus replication by blocking the M2- protein ion channel. There is experimental evidence that the amantadine blockade of the M2 channel occurs within the transmembrane (TM) region.

Binding of blockers to the Influenza A ion-channel within the TM region is studied using automated docking calculations. Our study suggests that studied cage compounds inhibit the M2 ion channel by binding to the His37 residue. The adamantane cage fits into a pocket formed by Trp41 residue, while the hydrogen bond is formed between hydrogen atom of ammonium nitrogen and the nitrogen of histidine residue. This finding is supported by an available experimental data and should help to obtain better understanding of the inhibition mechanism of the Influenza A M2 ion channel.

Based on the docking and QSAR studies, new potential bicyclo[2.2.1]heptane-derivatives were synthesized.

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Engineering of the NlaIV Restriction Endonuclease Sequence Specificity

Krzysztof Skowronek

Laboratory of Bioinformatics and Protein Engineering, International Institute of Molecular and Cell Biology, Trojdena 4, 02-109 Warsaw, Poland, krzysiek@genesilico.pl

Restriction endonucleases (RE) recognize their target sequences with unsurpassed specificity. Even single change in the recognition sequence results in decrease of the cleavage rate of at least five orders of magnitudes. In the same time members of this group recognize more than 260 different sequences. For those reasons they serve as a paradigm of nucleic acid sequence recognition by proteins. Due to the divergent evolution of this group of enzymes and strong evolutionary pressure ensuring high cleavage specificity it is extremely difficult to change recognition sequence of RE. Additional obstacle is necessity of protecting host from such cleavage by supplying DNA methyltransferase of the same (or broader) specificity.

We present preliminary results of engineering sequence specificity of RE NlaIV (GGN↓NCC). By selecting RE with degenerate recognition sequence we avoided problem of engineering methyltransferase since all subsets of the original recognition sequence are methylated by the wild type (wt) methyltransferase. Moreover such strategy does not attempt to change protein-DNA contacts already present in the wt enzyme but rather creates new ones involving central dinucleotide in the target sequence. After analysis of the experimentally validated structural model of NlaIV RE three regions with potential to develop new contacts with recognition sequence were selected. Several oligonucleotides carrying random substitutions of NNS sequence in 2-5 codons were synthesized via split-and-mix approach to control mutagenesis frequency at the codon level. Mutations were introduced in PCR reactions to linear molecules containing T7 promotor, coding sequence and T7 terminator flanked on each side by NlaIV recognition sequences differing in central dinucleotide. There were no other NlaIV recognition sequences throughout the rest of DNA molecules. Total of 5 different libraries were created. Each was subjected to iterative in vitro selection protocol consisting of compartmentalized in vitro transcription-translation reaction, removal of molecules containing terminal biotin added on one end during PCR reaction, PCR selection and amplification of molecules containing intact other end and recycling. After several rounds of such selection DNA molecules were cloned, screened for specificity changes and sequenced. Finally sequence preferences of selected variants were assayed on exhaustive panel of 10 substrates containing single NlaIV recognition sequence with different central dinucleotides. Several mutants appear to have selectivity toward central dinucleotide narrowed down to less than 4 (out of 10 possible) sequences. Our results suggest that presented approach of RE engineering may be successful in developing enzymes with novel specificities.

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Mutagenesis and Directed Evolution of Key Bacterial Monooxygenases toward Improved Biocatalysis

Thomas J. Smith,[1] Tim Nichol,[1] Ashlee Perry,[1] Elena Borodina,[2] Hélène Moussard[2] and J. Colin Murrell[2]

1 Biomedical Research Centre, Sheffield Hallam University, Owen Building, Howard Street, S1 1WB Sheffield, UK, t.j.smith@shu.ac.uk
2 Department of Biological Sciences, University of Warwick, Coventry, UK

The soluble diiron monooxygenases (SDIMOs), which catalyse the initial addition of oxygen to the growth substrate in many hydrocarbon-oxidising bacteria, have the potential for use in bioremediation and highly specific commercial oxygenation reactions. Among these enzymes, the remarkably versatile soluble methane monooxygenase (sMMO) can oxidise the very unreactive methane molecule to methanol (a more valuable fuel) and also co-oxidises a diverse range of more than 100 hydrocarbons and other uncharged molecules, which lends it to numerous potential applications in biocatalysis and bioremediation. The homologous alkene monooxygenase (AMO), by contrast, has a relatively narrow substrate range (mainly terminal and subterminal alkenes) but can be used to produce valuable chiral epoxides, including *R*-1,2-epoxypropane, which may be the smallest chiral metabolite in the natural world. Use of protein design and evolution methods to manipulate the unique properties of sMMO and AMO presents a particular challenge because neither enzyme has been successfully expressed at a useful level of activity in *Escherichia coli*. We previously developed novel expression systems using alternative bacterial hosts that allow sitedirected mutagenesis of both these enzymes and have prepared derivatives of AMO with enhanced enantioselectivity, one of which yields *R*-epoxypropane with an enantiomeric excess of 90 % compared to 79 % in the wild-type. In sMMO, mutagenesis was recently used to identify a residue (Leu 110) in the active centre of sMMO that is critical in determining the ability of the enzyme to orient aromatic substrates precisely in its active site. By further development of both expression systems, together with improved screening technology, we are now able to construct and screen libraries of quasi-random mutants of sMMO and AMO and select mutants for changes in enantioselectivity during epoxygenation of alkenes or expanded substrate range with aromatic compounds.

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Towards Biocatalysts for Organosilane Biotransformations

Radka Snajdrova and Gideon Grogan

Department of Chemistry, York Structural Biology Labortory, University of York, Heslington, YO10 5YW York, UK, radka@ysbl.york.ac.uk

The application of enzymes to the manipulation of classical organic atoms such as carbon, hydrogen, oxygen, phosphorus and sulphur is widespread; there has been scant reference to the transformation of silicon-containing substrates, or more specifically, enzymatic reactions at the silicon atom itself. This is surprising as silicon, the second most abundant atom in the earth's crust, is the object of a complex and varied biochemical reactions that in part contribute to the global silicon cycle.

The manufacture of silicon-based materials typically requires high temperatures, high pressures or the use of caustic chemicals. By contrast, the biological production of amorphous silica, the simplest siloxane is accomplished under mild physiological conditions, producing a remarkable diversity of exquisitely architectures, whose nanoscale structural complexity operates on a scale currently beyond that of the limits of contemporary engineering.[1]

We aim to investigate the possibility of using the undiscovered yet undoubted ability of natural biocatalysts to catalyze reactions at the silicon atom, for synthetically useful Si-O and Si-C bond cleavages.

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B Factor Iterative Test (B-FIT) for Enhancing Enzyme Stability in Organic Solvents

Pankaj Soni

Max-Planck-Institute of Coal Research, Group Prof. Reetz, Kaiser-Wilhelm-Platz 1, 45470 Mülheim An Der Ruhr, Germany, soni@mpi-muelheim.mpg.de

The temperature factor or B-factor can be thought of as a measure of how much an atom oscillates or vibrates around the position specified in an X-ray diffraction structure. B-factors are directly related to thermal motion and flexibility. Considering the B-factor of an amino acid in a protein structure, a high B-factor corresponds to a flexible amino acid residue while a low B-factor indicates a more rigid one. The design rules for increasing the organic solvent stability stresses two key issues: retaining the water bound to the protein which is essential for catalysis and to increase the inherent strength of the protein. B-FIT relies on the latter approach: doing iterative saturation mutagenesis (ISM) on amino acid residues having low B value to enhance rigidity while ISM on amino acid residues of high B value to decrease the rigidity of the enzyme.

Our previously successful efforts with B-FIT as a tool for enhancing the thermostability of *Bacillus subtilis* lipase A (BSL)1 and thermolability of *Pseudomonas aeruginosa* lipase (PAL)2 prompted us to expand the applicability of our approach to stability in organic solvent as well. In this study, we use the BSL (wild type) and five thermostable mutants IV (Met134Asp), VII (Met134Asp, Ile157Met), IX (Met134Asp, Ile157Met, Tyr139Cys, Lys112Asp), X (Met134Asp, Ile157Met, Tyr139Cys, Lys112Asp, Arg33Gln, Asp34Asn, Leu35Asp) and XI (Met134Asp, Ile157Met, Tyr139Cys, Lys112Asp, Arg33Gly) as well as PAL (wild type) and its thermolabile variant A obtained in our previous studies.[1,2] Different organic solvents like n-butanol, acetonitrile, dimethyl sulfoxide and dimethyl formamide were used in this study. We observed that for all the organic solvents tried, as the inherent strength of the protein molecule increases, the stability in organic solvent increases and vice versa. In fact, both BSL and PAL wild type have a half life of ten minutes in ACN (50%) while the most stable mutant XI has a half life of over 24h while the labile mutant of PAL is not even for 2 min stable in 50% (v/v) ACN. Our results clearly show the potential of using BFIT for evolving enzymes for their stability in organic solvents.

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Engineering a Beta-lactamase Allosterically Regulated by Kanamycin

Pascale Mathonet, Humberto Barrios, Alex Volkov, Jacques Fastrez and **Patrice Soumillion**

Unité BIOC, Institute of Life Sciences, Université Catholique de Louvain, Place Croix du Sud 4-5, box 3, 1348 Louvain-la-Neuve, Belgium, patrice.soumillion@uclouvain.be

Using the phage display technology, we have selected a beta-lactamase insertant which is activated by an aminoglycoside, kanamycin, at micromolar concentration. Surprisingly, the enzyme is also specifically inhibited by some anions such as 2-(4-morpholino)ethanesulfonate and the kanamycin activation effect is resulting from the release of this inhibition, anions and kanamycin appearing to be mutually exclusive. Regulation effects are mainly affecting the Km of the enzyme suggesting that kanamycin is modulating the equilibrium between an ensemble of fluctuating conformations of the enzyme. This hypothesis is evaluated through NMR experiments. Overall, these artificial regulatory properties are similar to what is observed in natural allosteric enzymes that often are both inhibited and activated by effectors acting on partially overlapping binding sites.

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Everything is There but it is Not a Beta-lactamase

Carole Urbach, Christine Evrard, Jacques Fastrez,
Jean-Paul Declercq and **Patrice Soumillion**

Unité BIOC, Institute of Life Sciences, Université Catholique de Louvain, Place Croix du Sud 4-5, box 3, 1348 Louvain-la-Neuve, Belgium, patrice.soumillion@uclouvain.be

It is largely accepted that serine beta-lactamases evolved from some ancestral D-Alanyl-D-Alanine-peptidases involved in the biosynthesis and maintenance of the bacterial peptidoglycan. DD-peptidases are also called penicillin-binding proteins (PBPs) since they form stable acyl-enzymes with β-lactam antibiotics such as penicillins. On the other hand, β-lactamases react similarly with these antibiotics but the acyl-enzymes are unstable and rapidly hydrolysed. Besides, all known PBPs and beta-lactamases share very low sequence similarities, thus rendering it difficult to evolve a PBP into a beta-lactamase by directed or random mutagenesis. We identified a new family of cyanobacterial PBPs featuring a high sequence similarity with the most widespread class A beta-lactamases. Interestingly, the omega-loop, which, in the beta-lactamases, carries an essential glutamate involved in the deacylation process, is six amino acids shorter and does not contain any glutamate residue. From this new family of proteins, we characterised PBP-A from *Thermosynechococcus elongatus* and discovered hydrolytic activity with synthetic thiolesters that are usually good substrates of DD-peptidases. Penicillinase activity is 7 orders of magnitude lower than for typical class A β-lactamase, which is typical for a PBP. In a first attempt to generate beta-lactamase activity, a 90 fold increase in deacylation rate was obtained by introducing a glutamate in position 158 of the shorter omega-loop. The crystal structures of the wild-type protein in the free and penicilloylated forms and of L158E mutant were solved, giving insights in the catalytic mechanism of the proteins. Since all the active site elements of PBP-A-L158E, including an essential water molecule, are almost perfectly superimposed with those of the TEM-1 enzyme, the question why our mutant is still far from being a penicillinase remains. Up to now, our additional attempts to further enhance PBP-A penicillinase activity, both by site directed and random mutagenesis approaches.

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Improving Pyranose 2-oxidase from *Trametes Multicolor* for Biocatalytic Applications by Genetic Engineering

Oliver Spadiut,[1] Christina Divne[2] and Dietmar Haltrich[1]

1 Department of Food Sciences and Technology, Division of Food Biotechnology, University of Natural Resources and Applied Life Sciences, Muthgasse 18, 1190 Vienna, Austria, oliver.spadiut@boku.ac.at
2 KTH Royal Institute of Technology, Stockholm, Sweden

Pyranose 2-oxidase (P2Ox) from *Trametes multicolor*, a homotetrameric flavoprotein, catalyzes the oxidation of different aldopyranoses at C2 to the corresponding 2-ketoaldoses, producing H_2O_2 as a by-product. The monosaccharide D-glucose is its preferred substrate, whereas D-galactose performs poorly with only 5.2% relative activity. Oxidation of D-galactose at position C2 is highly interesting from an applied point of view. The product obtained in the transformation, 2-keto-D-galactose, can be reduced easily at position C1 to yield D-tagatose, a ketose sugar with significant potential as a non-cariogenic, low caloric sweetener. In order to improve P2Ox with respect to its catalytic activity with the poor substrate D-galactose as well as thermal stability, we created different mutants by rational protein design, saturation mutagenesis and directed evolution, and combined different beneficial mutations to accumulate the single positive effects. One of the resulting mutants, L537W/E542K, did not just show a significant increase in its catalytic activity with both sugar substrates D-glucose and D-galactose, but also a considerable improvement of its stability as is evident from an increase of the melting temperature Tm by 17°C. The enhanced thermal stability was proven by DSC measurements, which also revealed an altered melting curve of this variant. In contrast to the wild-type enzyme, L537W/E542K denatures in two separate steps, suggesting the formation of a thermo-stable fragment of P2Ox. Analysis of the crystal structures of these variants suggests that improved interactions between the subunits of P2Ox are responsible for the remarkable increase in stability.

In order to further improve the catalytic activity of P2Ox with the poor substrate D-galactose as well as with alternative electron acceptors, we used saturation mutagenesis at position V546, located in the active site. Different variants, which showed increased activity with D-galactose in a high-throughput screening assay, were all identified as V546C. Turnover numbers for both D-glucose and D-galactose, as well as for alternative electron acceptors (e.g., benzoquinone) measured for this variant were significantly increased compared to the wild-type enzyme. By introducing another single mutation variant V546C/T169G was obtained, which showed almost identical kinetic constants for glucose and galactose. The usefulness of this mutant for possible food applications, i.e. the conversion of hydrolyzed lactose containing both D-glucose and D-galactose, was proven in small scale conversion experiments. In addition, this variant is quite attractive for applications in biofuel cells, since catalytic activities for the alternative electron acceptors 1,4-benzoquinone and ferricenium ion were increased 39 and 49 times, respectively.

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Biocatalyst Panels: Using Tunable Biocatalysts to Accelerate Process Development

Jeffrey L. Spelberg,[2] Gjalt W. Huisman[1] and Melanie Schürmann[2]

1 Codexis Inc., Redwood City, CA, USA
2 Codexis Inc., Jülich Chiral Solutions GmbH, Prof.-Rehm-Str. 1, 52428 Jülich, Germany,
jeffrey.lutjespelberg@codexis.com

Codexis has created panels of genetically diverse biocatalysts for use in pharmaceutical process research and development. A Codex™ biocatalyst panel is a set of functionally diverse enzyme variants that are pre-tuned to accept a wide range of substrates, produce different stereoisomers, be chemically robust and be manufactured on commercial scale. The plate variants are also designed to yield structure-function information that can be used to provide additional diversity. For example, screening of a pharmaceutically relevant ketone with commercially available ketoreductases showed no enzyme with desired activity. However, screening of the ketoreductase Codex™ biocatalyst panel plate identified multiple variants with a range of activities and enantioselectivities. Based on the sequence-activity data from this panel, a new plate of customized variants was designed that showed increased activity and enantioselectivity toward the desired enantiomer. This approach allows for quick determination of the feasibility of a biocatalytic route and provides a diagnostic tool for further optimization using directed evolution.

In our presentation we will show the development and application of Codex™ biocatalyst panels. The result of this new technology reduces the development time required to generate a suitable biocatalyst to perform a selected reaction.

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Evolution of Screening Assays during the Extensive Phenotypic Remodeling of an Unspecific Human Serine Protease towards Higher Activity, High Selectivity and Blood Serum Inhibitor Insensitivity

Michael Strerath, O. Hesse, N. Habrich, J. Tebbe, C. Votsmeier, A. Scheidig, P. Scholz, U. Beister, C. Götzberger-Schad, K. Baral, K. Odendahl, S. Haupt, N. Koch, S. Frings, H. Plittersdorf, A. Rüter, M. Maass, K. Kirchner, K. Meyer, K. Pellengahr, U. Gritzan, G. Zeidler, D. Kalb, V. Majewski, B. Selbach, Y. Störmer, N. Lobrenz, M. Rarbach, U. Kettling, A. Koltermann, T. von Rüden, U. Haupts and W. M. Coco

DIREVO Biotech AG, Nattermannallee 1, 50829 Cologne, Germany, michael.strerath@gmail.com

With advantages like irreversible target inactivation and catalytic turnover, human proteases represent a conceptual alternative to therapeutic antibodies. The presented data provide insights into a screening campaign in which the low specificity serine protease, human cationic trypsin, was optimized to specifically cleave and inactivate human Tumor Necrosis Factor alpha (TNF-alpha), a key cytokine in the development and progression of rheumatoid arthritis and other inflammatory disorders. In addition to specificity, a major program goal was improving resistance to the rapid-acting serpins and macroglobulins inhibitors which are present in human blood. The described screening campaign resulted in a phenotypically remodeled protease with fourfold higher specific activity against TNF-alpha than the starting protease, a hundreds-fold increase in selectivity, and which possesses several thousand-fold higher inhibitor insensitivity. The presented work will include the practical and theoretical considerations which rendered this highly complex, multi-parameter screening campaign successful. Because of the extensive improvements to the protease, we were over time able to switch from TNF-mimicking small peptide substrates to whole-TNF protein substrates and from extremely diluted concentrations of human serum to 100% human serum. Likewise, assay window and selection pressures were repeatedly adjusted, for example increasing the concentration of non-specific competitor proteins to further increase protease specificity. This combination of strategies, combined with high-throughput screening represents a powerful and general approach to protease engineering.

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Molecular Modeling of Substrates Recognition by Penicillin Acylase

Oleg V. Stroganov, Novikov F.N., Shoshina N.V., Chilov G.G. and Vytas K. Svedas

Department of Biokinetics, Belozersky Institute of Physico-chemical Biology, Moscow State University, Khokhlova st., 6, 119992 Moscow, Russian Federation, stroganov@belozersky.msu.ru

Penicillin acylases (PA) are industrially important enzymes standing for large scale production of antibiotic nuclei. Due to the excellent biocatalytic potential of these enzymes understanding of molecular mechanism of substrates recognition in the active site of PA is highly important.

Combination of molecular docking and molecular dynamics was used to depict the series of events coinciding binding of the native substrate of PA – penicillin G. It was found, that binding of the substrate is governed by reversed "opening" of residues Arg145A-Phe146A, which comprise bottleneck of the hydrophobic pocket of enzyme. Molecular modeling also revealed that non-productive binding plays an important role in substrates recognition and may influence overall catalytic properties of PA.

The proposed mechanism of substrate binding was evaluated and refined in study of enantioselectivity of PA. 26 systems containing L- and D-phenylacylated derivatives of aminoacids and aminoalcohols were modeled. Productive modes of substrates binding were found out after docking experiments and verified by 10-ns molecular dynamics simulations. For each system free energy of binding and relative reactivity were estimated and calculated enantioselectivity showed well agreement with experimental data. Detailed study of molecular dynamics trajectories of productive enzyme-substrate complexes revealed that interactions between substrates and polar residues of active site were maintained by a system of highly conservative bridging water molecules. Another important observation concerned non-productive binding of substrates. Molecular docking of phenylacylated aromatic aminoacids and their derivatives showed that together with correct productive pose, so called "reversed" conformations of substrate had high binding affinities. In these conformations the aromatic ring of aminoacids occupied hydrophobic pocket of the enzyme. Alternative modes of binding were tested by molecular dynamics, and it appeared that "reversed" conformations played essential role in the substrate binding mechanism in the case of aminoacids and aminoalcohols with bulky hydrophobic side chains. The complete model of the substrate recognition by penicillin acylase, which took into account both productive and non-productive binding, water bridges and statistics of near-attack conformations, was able to extend our understanding of substrates recognition by PA. The calculated binding affinities and estimated enantioselectivity values reproduced experimentally obtained data well, thus making ground for robust prediction of penicillin acylase substrate specificity *in silico*.

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Construction of a Library of Orthogonal Transcription Factors with their Corresponding Promoters Based on Design of Synthetic Zinc-finger Proteins

Maria Suarez, H. Garcia-Seisdedos, J. Carrera, G. Rodrigo, F. Pinto, D. Camsund, T. Landrain and A. Jaramillo

1 Laboratories Biochimie CNRS UMR7654, Ecole Polytechnique, Route de Saclay, 91128 Palaiseau, Cedex, France, maria.suarez@polytechnique.edu
2 Departamento de Quimica Fisica, Facultad de Ciencias, Universidad de Granada, 18071-Granada, Spain
3 Present address: Department of Chemistry and Biochemistry and Center for Biomolecular Structure and Organization, University of Maryland, College Park, Maryland 20742, USA

A large limitation for the design of more complex biological circuit is the small amount of orthogonal transcriptional regulators available. We propose the construction of a new type of library of true orthogonal transcription factors with their corresponding promoters based on design of synthetic zinc-finger proteins (ZFPs) that are inhibitors. We aim to use the propriety of ZFPs binding DNA for blocking the RNA polymerase by steric competition on the promoter sequences. ZFPs are the most common DNA-binding motifs found in eukaryotes and also have been identified in prokaryotes. ZFPs are known to be composed of modules that bind to 3-nucleotids motifs in a very specific manner, we used this property to create the recognition sequences by computational methods and then we constructed the corresponding ZFP modules. We used modular assembly of 4 ZFP retaining sequence and we only considered ZFPs with recognition sites of the form GNN triplets that are the most specific. We looked for all possible operators of 12 bases (4 times GNN) that could be synthesized and we matched the list with the *E. coli* genome in order to look for orthogonality. We finished with a list of 17 sequences remaining that we have aligned and put into clusters in order to maximize the difference between them. The last step will be to test in vivo the ZFPs with their corresponding promoter sequences in order to quantify their level of repression and thanks to ChIP on chip experiments and proteomics their orthogonality to the chassis and between each others. This work will permit to enlarge already existing libraries of promoters such as the Registry of Biobricks proposed by the MIT and will deeply facilitate the creation of more complex biological circuits that call for higher numbers of orthogonal genes without cross-talks.

P 80

Bioinformatic Analysis of Penicillin Acylase Family Reveals Key Residues Essential for Enzyme Stability

Dimitry A. Suplatov,[1] Dorel T. Guranda[2] and Vytas K. Svedas[1,2]

1 Faculty of Bioengineering and Bioinformatics, Lomonosov Moscow State University, Leninskiye Gory, building 73, 119992 Moscow, Russian Federation, genesup@belozersky.msu.ru
2 Belozersky Institute of Physico-Chemical Biology

Penicillin acylases (PAs, EC 3.5.1.11) represent a group of industrially important enzymes widely used for production of semi-synthetic antibiotics. Here, a variety of computational techniques has been employed to examine the most conserved relatives among penicillin acylases from *Escherichia coli* (EcPA) and *Kluyvera citrophila* (KcPA). All protein that belong to penicillin acylase family (entry IPR002692 in the InterPro database) were considered to generate the multiple sequence alignment (MSA). From each cluster of sequences that were more than 85% identical only one sequence was retained. Only sequences with at least 0.25 bits score per column with KcPA sequence were accepted into the evolving MSA, ensuring that the sequences in the alignment are not too distantly related and important residues will be conserved. T-Coffee, Clustalw, ProbCons and Mafft programs were then used to compile the final MSA. Eventually, conserved residues were assigned through statistical comparison of final MSA with neutral simulated model. One possible explanation for stability discrepancy between PAs could be different quantity and quality of molecular interactions especially ion bridges between Arg/Lys and Asp/Glu. Modeller and Gromacs packages were used to model the structure of KcPA. Acquired representation of KcPA and PDB structure of EcPA were used to examine the variations in the extent of the ion-pair networks. Interactions observed at least 80% of time of molecular dynamics simulation were considered. It has been observed that single amino acid insertion in KcPA sequence versus EcPA alters a minor structural change. Hence ArgB291 is shifted to more favorable position where it becomes capable of interacting with GluB327, AspB325 and GluB265. We consider ArgB291 switch important for discrimination between KcPA and EcPA stability. Firstly, because it is located in close vicinity to the active site. Secondly, considering different pKa of Asp and Glu, ArgB291 in KcPA has the capability of forming two ion interactions gradually during the pH decrease. This could enhance enzyme stability in acidic conditions. Finally, ArgB291 and GluB327/AspB325 are classified to different structural CATH domains. At the same time neighboring ArgB295 forms a contact with GluB265 in KcPA as well as in EcPA while both acids are marked as conserved in the EcPA phylogenetic clade. This interaction could potentially be important for both enzymes.

Stability and activity of KcPA has been investigated. The effect of temperature and pH on the activity of KcPA concurs with observations reported for EcPA. Contrary, investigation of the KcPA inactivation kinetics has outlined a considerable difference with EcPA. KcPA has been found significantly more stable in acidic conditions. Considering all the observed experimental dependencies we back the suggestion that native stable PA conformation is maintained by several critical salt bridges most likely formed by guanidinium group of arginine, amino group of lysine and acidic groups of aspartate and glutamate.

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Molecular Modeling of Nucleophile Binding and Reactivity in Penicillin Acylase-catalyzed Acyl transfer

Vytas K. Svedas

Faculty of Bioengineering and Bioinformatics, Lomonosov Moscow State University, Vorobjev Hills 1, 119991 Moscow, Russian Federation, vytas@belozersky.msu.ru

Binding of the nucleophile by penicillin acylase (PA) and its reactivity are key factors in PA-catalyzed acyl transfer reactions, which are of special interest due to the practical importance of the biocatalytic synthesis of β-lactam antibiotics and chiral acylation of amino compounds in aqueous medium. However, relatively low affinity of PA towards nucleophiles hampers structural studies of the nucleophile binding subsite. Molecular modeling was applied to get the insight into nucleophile binding by PA and to set the quantitative basis for prediction of nucleophile reactivity in PA-catalyzed acyl transfer. By performing molecular docking followed by molecular dynamical (MD) simulations in explicit aqueous solution four acylenzyme-nucleophile complexes have been studied: formed by two acylenzymes (phenylacetyl-PA or D-phenylglycyl-PA) with two β-lactam antibiotic nuclei as nucleophiles (6-aminopenicillanic or and 7-aminodesacetoxycephalosporanic acid). MD simulations were crucial to refine the structure of the acylenzyme-nucleophile complexes obtained by conventional molecular docking. Moreover, MD simulations of acylenzyme-nucleophile complexes and free acylenzymes in aqueous solution allowed to perform quantitative comparison of nucleophile and water reactivity by collecting statistics of near attack conformations from the MD trajectories. Correspondence of experimentally measured nucleophile reactivities to the calculated values confirmed validity of the suggested computational approach for the evaluation of such a crucial parameter of the biocatalyst as its synthetic potential.

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Rational Redesign of *Candida antarctica* Lipase B for Kinetic Resolution of Bulky Sec-alcohols

Per-Olof Syrén, Michaela Edin and Karl Hult

Department of Biochemistry, School of Biotechnology, Royal Institute of Technology, AlbaNova University Center, 106 91 Stockholm, Sweden, per-olof.syren@biotech.kth.se

Lipase B from *Candida antarctica* (CALB) contains a stereoselectivity pocket that dictates the enantiopreference of secondary alcohols.[1] CALB is of industrial interest since it is stable in organic solvents, at high temperatures and displaying high selectivity. A major drawback of using CALB in resolution of secondary alcohols is the somewhat stringent requirements on the substrate structure. The large substituent must be larger than ethyl and the medium substituent must be an ethyl group or smaller.

Modelling studies showed that the bigger pocket introduced by the mutation W104A could accommodate more sterically hindered substrates. It has previously been shown by our group that the mutant enzyme W104A displays high activity towards symmetrical sec-alcohols.[2] The enantioselectivity was shifted from R to S using 1- phenylethanol as acyl acceptor in kinetic resolution (KR).[1]

The performance of the W104A biocatalyst in KR of various bulky sec-alcohols has been evaluated.

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Rational Design of *Candida antarctica* Lipase B for the Ring Opening Polymerization of Lactides

Mohamad Takwa, Mats Martinelle and Karl Hult

Royal institute of technology, Faculty of Biotechnology, Department of Biochemistry, Albanova university center, 106 91 Stockholm, Sweden, takwa@biotech.kth.se

Polylactides (PLAs) are known to be biocompatible and biodegradable polymers. They found use in materials devices, medical applications and plastics.

The enzymatic polymerization of lactides is dependent on the configuration of the secondary alcohol at the propagating chain end. Due to the enantioselectivity of lipase B from *Candida antarctica* (CALB), the L,L-lactide do not polymerize while the polymerization of the D,D-lactide was very slow. This low activity of the enzyme towards the enantio-preferred monomer is believed to be due to unfavorable steric interactions in the active site caused by the methyl groups of both the acyl donor and acceptor. In order to get a molecular understanding of monomer-lipase interaction and to design a more active enzyme toward the D,D lactide, a molecular modeling study was done. With the help of molecular dynamics, rational design by point mutations is in progress on CLAB.

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Protein-solvent Interaction and Simulation Studies of Solvent Stable and Thermostable Lipase from *Bacillus* strain 42 in Water-solvent Mixtures

Tengku Haziyamin Abdul Hamid,[1] Muhammad Aliff Mohd Latif,[3] Raja Noor Zaliha Raja Abd. Rahman,[1] Mahiran Basri[3] and Abu Bakar Salleh[2]

1 Department of Microbiology, Faculty of Biotechnology and Biomolecular Sciences, University Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia, haziyamin@iiu.edu.my
2 Department of Biochemistry, Faculty of Biotechnology and Biomolecular Sciences, University Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia
3 Department of Chemistry, Faculty of Science, University Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia

A purified solvent stable and thermostable recombinant lipase, Lip 42, isolated from *Bacillus* sp. strain 42 was previously shown to be stable in polar organic solvents such as dimethyl sulfoxide (DMSO) and methanol. Stabilities in different solvent compositions were studied based on 40°C pre-incubation in solvent and the purified lipase was shown to retain at least 100% residual activity in up to 45% v/v DMSO and 45% v/v methanol. In 60% v/v DMSO, 68% of residual activity was retained, however, this dramatically reduced to 6.5 % at 65% v/v DMSO. Activity enhancement was recorded at lower solvent composition (less than 45% v/v solvent), whereby, at 30% v/v DMSO, enhancement was recorded to be as much as 35%. Enhancement tends to increase as temperature increases. Based on these solvent stability margin, molecular dynamic simulations were then carried out in the presence of water, 60% v/v DMSO + 40% v/v water and 100% v/v DMSO, by using a structure model predicted from a highly homologous (97%) lipase (PDB:1JI3). Results showed that the Lip 42 structure was retained and the flexibility of polypeptide backbone decreased or increased depending on the location of loop regions. Flexibility changes in the helix-loop-helix-motif covering catalytic triad were found to be associated with a hydrophobic cluster region. The presence of 60% v/v DMSO resulted in the disorganization of the cluster, accompanied with non-native H-bonds formations. However, the cluster still presents in 100% v/v DMSO and resembles to that of water simulation. Mutant form of lip 42 V174S contains residue substitution near the cluster and within helix-loop helix motif. At 50°C pre-incubation, the mutant lost as much of high temperature enhancement commonly observed in low DMSO composition. This indicates the potential role of hydrophobic residues in helix-loop-helix motif and the cluster in interfacial activation.

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Stability and Activity Constrain Evolution of an Antibiotic Resistance Enzyme

Veena L. Thomas, Andrea C. McReynolds and Brian K. Shoichet

Department of Pharmaceutical Chemistry, University of California, San Francisco, 1700 4th Street, Room N508, Campus Box 2250, 94158-2550 San Francisco, CA, USA, veenathomas@alum.mit.edu

Antibiotic use has led to the evolution of antibiotic resistance enzymes able to hydrolyze newer antibiotics, leaving them useless against killing bacteria. Are there biophysical costs to evolving drug resistance? Can we exploit these biophysical costs to minimize emergence of antibiotic resistance? I am studying these questions in the enzyme betalactamase, the major resistance mechanism against beta-lactam antibiotics like penicillin.

Enzyme structures reflect tradeoffs against several orthogonal constraints. Ligand recognition is often counterproductive to stability: the enzyme must leave key interactions unfulfilled to be satisfied upon ligand-binding. This leads to high-energy interactions in active sites, as enzymes cluster like-charged residues and expose hydrophobic areas to water. To maintain its catalytic activity, an enzyme must sacrifice some potential stability it could achieve with another, inactive sequence. How does the stability-function hypothesis relate to evolution of new properties? In order to investigate the biophysical consequences of evolving new substrate spectra, extended spectrum AmpC beta-lactamase mutants were characterized.

Preliminary results suggest that though these enzymes have acquired 100-200-fold increased activity against thirdgeneration cephalosporin antibiotics, they have decreased thermodynamic stability – from 1.7 to 4.1 kcal/mol - and decreased activity against their native substrates from 5-400-fold. In order to determine the molecular bases for the gain of activity and loss of stability, four structures of drug-resistant mutant enzymes were determined by x-ray crystallography. V298E disrupts a minihydrophobic core 20 Angstroms away from the catalytic serine; though this significantly destabilizes the protein, the structure of this enzyme to 2.6 Angstrom resolution reveals that it causes a “domino effect” of structural changes, resulting in a loop forming one wall of the binding site to flip outward. Similarly, the 1.6 Angstrom resolution structure of a mutant enzyme containing an insertion in the “omega” active site loop reveals that it also results in a new conformation of this loop, active site enlargement, and thus subsequent activity against bulky antibiotics. Comparison of the apo and holo structures of the antibiotic-resistant mutant T70I to 2.1 Angstrom resolution suggest that its evolution of new function is related to an increase in enzyme flexibility and dynamics; this substitution causes the “omega” active site loop to become disordered in the apo form, increasing active site volume, though reordering in the presence of an active site inhibitor. These results suggest that both protein stability and native catalytic activity may constrain evolution of new enzyme functions, and suggest strategies to minimize evolution of future drug resistance.

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Comprehensive Evolutionary Analysis, Comparison of Active Sites and Re-classification of the SPOUT Superfamily of Methyltransferases

Karolina L. Tkaczuk

IIMBC, Bioinformatics & Protein Engineering, ks.Trojdena 4, 02109 Warsaw, Poland, poison-ivy@genesilico.pl

SPOUT methyltransferases (MTases) are a large class of S-adenosyl-L-methionine-dependent enzymes that exhibit an unusual alpha/beta fold with a very deep topological knot. In 2001, when no crystal structures were available for any of these proteins, Anantharaman, Koonin, and Aravind identified homology between SpoU and TrmD MTases and defined the SPOUT superfamily. Since then, multiple crystal structures of knotted MTases have been solved and numerous new homologous sequences appeared in the databases. However, no comprehensive comparative analysis of these proteins has been carried out to classify them based on structural and evolutionary criteria and to guide functional predictions.

We carried out extensive searches of databases of protein structures and sequences to collect all members of previously identified SPOUT MTases, and to identify previously unknown homologs. Based on sequence clustering, characterization of domain architecture, structure predictions and sequence/structure comparisons, we redefined families within the SPOUT superfamily and predicted putative active sites and biochemical functions for the so far uncharacterized members. We have also delineated the common core of SPOUT MTases and inferred a multiple sequence alignment for the conserved knot region, from which we calculated the phylogenetic tree of the superfamily. We have also studied phylogenetic distribution of different families, and used this information to infer the evolutionary history of the SPOUT superfamily.

We present the first phylogenetic tree of the SPOUT superfamily since it was defined, together with a new scheme for its classification, and discussion about conservation of sequence and structure in different families, and their functional implications. We identified four protein families as new members of the SPOUT superfamily. Three of these families are functionally uncharacterized (COG1772, COG1901, and COG4080), and one (COG1756 represented by Nep1p) has been already implicated in RNA metabolism, but its biochemical function has been unknown. Based on the inference of orthologous and paralogous relationships between all SPOUT families we propose that the Last Universal Common Ancestor (LUCA) of all extant organisms contained at least three SPOUT members, ancestors of contemporary RNA MTases that carry out m1G, m3U, and 2'O-ribose methylation, respectively. In this work we also speculate on the origin of the knot and propose possible 'unknotted' ancestors. The results of our analysis provide a comprehensive 'roadmap' for experimental characterization of SPOUT MTases and interpretation of functional studies in the light of sequence-structure relationships.

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Characterization of Site-specific Interactions of the MTGase Enzyme with G-CSF: Why is Q134 PEGylated and Q131 is Not?

Maria Valentini

CRS4, Bioinformatica, Edificio 1, Parco Scientifico e Tecnologico, Sardegna Ricerche, 09010 Pula, Italy, maria@crs4.it

We have investigated the usage of the MTGase enzyme (E.C.:2.3.2.13, PDBid:1IU4) from *Streptoverticillium mobaraense* for enzymatic PEGylation of therapeutic proteins as to prolong their circulating halflife, improve their stability and solubility and reduce their enzymatic degradation and immunogenicity.[1]

In this work we analyse the specific interactions of MTGase with Granulocyte-Colony-Stimulating-Factor (GCSF, PDBid:2d9q) by means of Molecular Dynamics (MD) and docking calculations, in order to rationalize our experimental data on the mono-PEGylation site of the native protein and of site specific mutants. We have experimentally demonstrated that MTGase interacts and reacts with G-CSF at the level of residue Gln 134 only, although 17 other potentially transglutaminable Gln residues are present. In particular, the neighbouring glutamine residue Gln131, which is part of the same flexible loop to which Gln 134 belongs, is not a substrate for the enzyme.

A question therefore arise: why is Gln 134 transglutaminated while Gln 131 is not? To answer this question we studied the dynamics of solvated G-CSF using the Gromacs[2] molecular dynamics package. We used Molecular Dynamics data to evaluate Root Mean Squared Fluctuations (RMSF) of G-CSF alpha-carbons to identify the most flexible zones along the backbone and inspected the dihedral angles of the two glutamine residues during the course of the simulation. We also performed large scale docking using RosettaDock[3] looking for evidence of interaction of Gln 134 and Gln 131 with the MTGase active site. Results show that Gln134 can assume different local conformations, while Gln131 is located in a more rigid region and we believe that this is the reason why it is not a substrate of MTGase.

Results from docking support those findings, given that distance-constrained large-scale RosettaDock calculations found a cluster of decoys where Gln 134 is located at the active site of MTgase. The same protocol with analogous constraints did not produce any correctly docked configuration. These results support the hypothesis that Gln 131 is not sufficiently flexible to be able to interact with the active site of MTGase.

Our results stress the importance of substantial flexibility for glutamine residues that are substrates of MTGase. Further work on the interaction of MTGase with other proteins is underway to assess how general is this conclusion.

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A Most Promiscuous Enzyme: Six Reactions Catalyzed by One Active site

Bert van Loo, Stefanie Jonas, Ann C. Babtie, Marko Hyvonen and Florian Hollfelder

Department of Biochemistry, University of Cambridge, 80 Tennis Court Road, CB2 1GA, Cambridge, UK, bv223@mole.bio.cam.ac.uk

We report an unusually promiscuous enzyme that is able to catalyze the hydrolysis of in total six different substrate classes. Originally assigned as a phosphonate monoester hydrolase (PMH) substantial second order rate accelerations ($(k_{cat}/K_m)/k_w$), ranging from 10e7 to as high as 10^{19}, are observed for the hydroplysis of phosphate mono- di- and triesters, phosphonate monoesters, sulfate monoesters and sulfonate monoesters. This collection encompasses a range of substrate charges between 0 and -2, transition states of a different nature, attack at two different reaction centres (P and S), and diverse intrinsic reactivities (half lives between 200 days and 0.5 million years under near neutral conditions). Catalysis occurs in spacious active site and involves a formylglycine nucleophile coordinated to a metal ion. Based on its crystal structure, PMH belongs to the alkaline phosphatase (AP) superfamily. Remarkably it encompasses many of the cognate activities previously observed for this superfamily. Our data suggests that PMH is an efficient generalist enzyme hat is not liable to the usual trade-off between activity and selectivity. Such reactive generalists may be useful all-purpose scavengers and can be presumed to have played a role in functional evolution by gene duplication.

Preliminary results from testing the promiscuity in a number of closely related PMHs and arylsulfatases (AS) shows that they all catalyze the cognate conversions of the AP-superfamily with high second order rate accelerations ($(k_{cat}/K_m)/k_w$) larger than 10^{10}). Not only does this confirm their relatedness, it also suggests that catalytic promiscuity is a widespread phenomenon in the AP-superfamily.

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Substrate Promiscuity and Directed Evolution of a Nonribosomal Peptide Synthetase Adenylation Domain (TycA)

Benoit Villiers and Florian Hollfelder

Department of Biochemistry, University of Cambridge, 80 Tennis Court Road, CB21GA Cambridge, UK, bv228@cam.ac.uk

Nonribosomal peptide synthetases (NRPSs) are modular biosynthetic systems that produce bioactive peptides with an activity spectrum ranging from antibiotic to antitumor activities. Each module is responsible for the incorporation of a specific amino acid into the final peptide and is composed of catalytically independent domains. The adenylation domain (A-domain) specifically recognises and activates the amino acid. It represents the main selectivity filter in NRPSs, and changing its specificity might allow to produce new bioactive peptides by combinatorial biosynthesis. In our project, the initiation module of the tyrocidine biosynthetic system (TycA) is used as a model NRPS A-domain to investigate A-domains substrate promiscuity, and the approach of altering substrate specificity by directed evolution. We reported[1] a 96-well format procedure for measuring adenylation activity of NRPS A-domains by the established ATP/[32P] pyrophosphate exchange assay. This format allows the measurement of approximately 384 data points per day. It can produce high quality kinetic data over 6 orders of magnitude in catalytic efficiency. This large dynamic range was exploited[2] to probe comprehensively TycA Adomain substrate promiscuity i.e. Michaelis-Menten parameters were determined for 30 substrates. The data define which substrate features contribute to efficient turnover. Hydrophobicity plays a major role in recognition of promiscuous substrates: we observe a broad correlation between the hydrophobicity of the substrate side chains (measured by logP) and $\log(k_{cat}/K_m)$ or $\log(K_m)$. The systematic mapping of substrate specificity suggests that Adomains are adaptable to accommodating alternative substrates, and provides a basis for exploring possible paths for future directed evolution of A-domains specificity. A directed evolution library screening procedure in a 96-well format is currently carried out to switch TycA A-domain substrate specificity from L-Phe (natural substrate) to L-Thr (showing a 106-fold lower catalytic efficiency). Three rounds of successive saturation mutagenesis at the 8 binding pocket positions, followed by recombination of beneficial mutations, have been carried out so far, giving a 100-fold increase in catalytic efficiency. Simultaneous saturation mutagenesis at two binding pocket positions is currently carried out to increase conformational freedom.

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The Alpha-galactosidase Type A Gene from *Aspergillus niger* Encodes a Fully Functional α-N-acetylgalactosaminidase

Lenka Weignerova,[1] Natalia Kulik,[2] Petr Pompachm,[3,4] Petr Novak,[4] Hynek Mrazek,[2,3] Karel Bezouska,[3] Vladimir Kren[1] and Rüdiger Ettrich[2]

1 Institute of Microbiology, Center for Biotransformation and Biocatalysis, Academy of Sciences of the Czech,Republic, Videnska 1083, 142 20 Praha 4, Czech Republic
2 Laboratory of High Performance Computing, Institute of Systems Biology and Ecology, Academy of Sciences of the Czech Republic, and Institute of Physical Biology, University of South Bohemia, Zamek 136, 373 33 Nove Hrady, Czech Republic
3 Department of Biochemistry, Faculty of Science, Charles University, Albertov 2030, 12840 Prague 2, Czech Republic
4 Institute of Microbiology, Laboratory of Molecular Structure Characterization, Academy of Sciences of the Czech Republic, Videnska 1083, 142 20 Praha 4, Czech Republic

Alpha-N-acetylgalactosaminidase (EC 3.2.1.49) is an exoglycosidase specific for the hydrolysis of terminal alphalinked N-acetylgalactosamine in various sugar chains, as e. g. blood group carbohydrate determinates. A large screening study of extracellular alpha-N-acetylgalactosaminidase activity of a library of filamentous fungi (42 strains) identified good producer - *Aspergillus niger* CCIM K2. The enzyme responsible for the activity was purified, biochemically characterized and N-terminal sequenced. Additional sequencing by mass spectrometry led to the conclusion that the protein is encoded by the gene aglA. Structural models of both enzymes encoded by the genes described in the genome as alpha-galactosidases A and B variants (genes aglA and aglB) were constructed and revealed significant differences of the active centers, which contributes to the understanding of the specificity of the hydrolyzed carbohydrates. Substrate docking clearly demonstrates the preference of the identified enzyme for alpha-D-N-acetylgalactosamine over galactose thus proving that the alpha-galactosidase type A gene from *Aspergillus niger* encodes a fully functional alpha-N-acetylgalactosaminidase.

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Novel Tandem Reactions and the Directed Evolution of a Promiscuous Bifunctional Enzyme

Sarah Whitehead and Nick Turner

Manchester Interdisciplinary Biocentre, University of Manchester, Oxford Road, M13 9PL Manchester, UK, sarah.whitehead@postgrad.manchester.ac.uk

The current text book description of enzyme catalysis relies upon high specificity. It has been noted for some time however, that several enzymes demonstrate promiscuous activities. This research aims to investigate the possibility of engineering promiscuous flavin dependent hydroxynitrile lyase (FAD-HNL) activity into the related enzyme aryl alcohol oxidase (AAO) and *vice versa*. In doing so a promiscuous bifunctional enzyme capable of catalysing the two step conversion of alcohols to commercially valuable cyanohydrins will be created. Such an enzyme will provide insight into the evolution of these two enzymes, provide information on their mechanisms of catalysis and also be commercially valuable. Molecular modelling was used to identify potentially important amino acid residues within the active sites of the two enzymes to be targeted for mutagenesis. These mutants have been produced using site directed mutagenesis. Although the production of active wild-type AAO in *Escherichia coli* has not been possible, the production of active recombinant wild-type FAD-HNL has been optimised and enables the production and analysis of mutant enzymes. In addition liquid phase screening assays for both activities have been developed.

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Improvement of Endochitinase Activity by Directed Evolution

Montarop Yamabhai

Department of Biotechnology, Faculty of Agricultural Technology, Suranaree University of Technology, 111 University Avenue, Meung district, 30000 Nakhon Ratchasima, Thailand, montarop@sut.ac.th

Chitinases (EC.3.2.1.14) comprise a group of enzymes that hydrolyze chitin by cleaving its b-1,4 N-glycosidic bond. Endochitinase cleaves randomly at internal sites of chitin, generating soluble low mass multimers of GlcNAc such as chitotetraose, chitotriose, and chitobiose. Chitinase has potential in various industrial applications, including degradation of chitin polymer into chitooligosaccharides of different lengths, which are useful in food and pharmaceutical industries. In Thailand, crustacean (prawn, crab, shrimp, and lobster) productions are one of the main agricultural products. Traditional methods for chitin waste disposal include landfill, incineration, and ocean dumping. These methods are costly, inefficient, and most importantly harmful to the environment. An alternative method is using enzyme. Since the process of extracting chitin from crustacean require the use of HCl to extract mineral and NaOH to degrade proteins, thus, obtaining chitinase that is active at acidic or alkaline condition will be beneficial for industrial application. The purpose of this study is to use directed evolution technology to try to improve the activity of this enzyme at different conditions. The endochitinase genes from *Bacillus licheniformis*, which are used extensively in biotechnology industry, have been isolated and expressed in *Escherichia coli*. A library of mutant enzymes has been constructed by a combination of error-prone PCR and DNA shuffling method. The protocol for high-throughput screening of active chitinase at various reaction conditions has been established. From a medium-throughput screening, we were able to isolate one mutant that demonstrated improved catalytic activity. When using chitobiose as substrate, the k_{cat}/K_m of an improved chitinase was approximately two fold higher than that of the wild type at both pH 3.0 and pH 6.0. Structure analysis revealed that the mutated residues are located in the catalytic and FNIII domains. These mutants could not be predicted by rational design. Thus, this approach can be used to screen for suitable chitinases for various applications in the future.

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Site Saturation Mutagenesis of Crucial Residues in NAD / NADP Specificity of Formate Dehydrogenase from *Candida methylica*

Emrah Yelboga

Molekuler Biyoloji ve Genetik Bolumu, Istanbul Teknik Universitesi, Ayazaga Kampusu, Maslak, 34390 Istanbul, Turkey, yelboga@itu.edu.tr

NAD^+-dependent formate dehydrogenase (EC 1.2.1.2, FDH) is the last enzyme in the metabolism of methanol in methylotrophs and catalyzes the oxidation of formate anion into carbon dioxide concomitant with the reduction of NAD^+ to NADH. One crucial role of this enzyme in industrial redox chemistry is to regenerate NADH from NAD^+- and formate. However, it uses only NAD as a coenzyme. It would be also desirable to regenerate NADPH by using NADP as a coenzyme. In previous experiments it was observed that adjacent amino acid residues Asp195, Tyr196 and Gln197 are responsible for binding of cofactor NAD. In order to change the cofactor specificity of formate dehydrogenase we have used saturation mutagenesis in a different manner. Mutant Asp159Ser cmFDH able to use NADP as a coenzyme.[1] However, this mutant binds NADP very weakly. In this study, we explore further mutations in the coenzyme binding domain to improve the Km of cmFDH for NADP. Saturation mutagenesis is the method of choice because it provides randomization of residues 195, 196 and 197. Random mutant libraries for each residues as well as triple mutant libraries were created seperately. Each single codon mutation libraries ensure effectiveness of site-saturation mutagenesis. Primary screening of single codon mutant libraries (32 different clones) allows discrimination of mutations with positive and negative effects. Identification of possible mutations will lead to combination of mutations with better catalytic activity for NADP.

The designed mutant proteins are produced and purified. Kinetic studies have been carried out and the results have been compared with the wild type cmFDH protein. The results suggest that the residues in these positions are major determinant of coenzyme specificity in NAD/NADP-dependent dehydrogenases.

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Selective Oxidation of Primary and Secondary Alcohols Using Variants of Galactose Oxidase

Bo Yuan, Franck Escalettes, Avgousta Ioannou and Nicholas Turner

School of Chemistry, University of Manchester, Oxford Road, Manchester, M13 9PL Manchester, UK, bo.yuan@postgrad.manchester.ac.uk

The development of catalysts for the selective oxidation of alcohols to aldehydes and ketones under mild conditions represents a major challenge in organic synthesis. Recently we have shown that variants of the enzyme galactose oxidase are capable of oxidising a wide range of chiral secondary alcohols with high enantioselectivity.[1] These variants also possess broader substrate specificity towards a range of primary alcohols. The aim of this project is to characterise these variants in more detail and specifically to develop efficient methods for the deracemisation of racemic secondary alcohols.

Deracemisation of racemic secondary alcohols can in principle be achieved by carrying out enantioselective oxidation using galactose oxidase with concurrent non-selective reduction of the ketone product. The major goal of this project is to identify conditions under which we can combine the enantioselective oxidation step and a nonselective reduction step to produce enantiomerically pure secondary alcohols. To achieve this, the reducing agent has to be compatible with the enzyme, which means ideally it needs to be active at pH 7-8, soluble in water, and active at room temperature. A range of reducing agents are currently being examined including sodium borohydride, borane-pyridine complex, sodium cyanoborohydride, borane tert-butylamine complex, borane ammonia complex, sodium trimethoxy borohydride and borohydride on polymer support.

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Oxidation of Human Cytochrome P450 1A2 Substrates by *Bacillus megaterium* Cytochrome P450 BM3: Humanized Bacterial Monooxygenase System

Chul-Ho Yun, Dong-Hyun Kim and Keon-Hee Kim

School of Biological Sciences and Technology and Hormone Research Center, Chonnam National University, Gwangju 500-757, Republic of Korea, chyun@jnu.ac.kr

Cytochrome P450 enzymes (P450s or CYPs) constitute a large family of enzymes that are remarkably diverse oxygenation catalysts found throughout nature, from archaea to humans.[1] Because of their catalytic diversity and broad substrate range, P450s are attractive as biocatalysts in the production of fine chemicals, including pharmaceuticals.[2] In spite of the potential use of mammalian P450s in various biotechnology fields, they are not suitable as biocatalysts because of their low stability, catalytic activity, and availability. Recently, wild-type and mutants of bacterial P450 BM3 (CYP102A1) have been found to metabolize various drugs through reactions similar to those catalyzed by human P450s.[3] It has therefore been suggested that CYP102A1 may be used to produce large quantities of the metabolites of human P450-catalyzed reactions. In this report, we show that the oxidation reactions of phenacetin, ethoxyresorufin, and methoxyresorufin, typical human P450 substrates, are catalyzed by both wild-type and mutant forms of CYP102A1. However, 7,8-benzoflavone, a typical inhibitor of human CYP1A2, showed distinct effects on the CYP1A2 catalyzed reactions dependent on the substrate. In the case of phenacetin, CYP102A1 enzymes show only O-deethylation product although two major products are produced as a result of Odeethylation and 3-hydroxylation reactions by human P450 1A2. Similar noncompetitive intermolecular kinetic deuterium isotope effects were observed for phenacetin O-deethylation both in the CYP102A1 and human CYP1A2 system. These results suggest that there is a common mechanism for the O-deethylation reaction catalyzed by both the bacterial CYP102A1 and human P450 enzymes. These results demonstrate that CYP102A1 mutants catalyze the same reactions as human CYP1A2. Taken together, it can be suggested that bacterial CYP102A1 can be used as a prototype enzyme for the industrial application of human P450 activities.

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Directed Evolution of a Hyperthermophilic Esterase from the Archaeon *Aeropyrum pernix* K1 to Improve the Hydrolysis Efficiency towards Plant Cell Wall

Guirong Zhang,[1] Jin Tao,[2] Zhenyan Jiang,[1] Yingying Xie,[1]
Chunyu Zheng,[1] Lirong Teng[1] and Shugui Cao[2]

1 College of Life Science, JiLin University, Changchun 130012, China, zgr@jlu.edu.cn
2 Key Laboratory for Molecular Enzymology and Engineering of Ministry of Education, Jilin University, Changchun 130023, China

The hyperthermophilic esterase from the archaeon *Aeropyrum pernix* K1 (APE1547) has the hydrolytic activity for a wide range of substrates. Ferulic acid esterase (FAE) is a fungal esterase, which can hydrolyze methyl ferulate, oligosaccharides ferulate and polysaccharides ferulate, and thus break the cross-linking between polysaccharides and lignin in plant cell wall. Most of FAEs have the same catalytic triad with APE1547 (Ser, His and Asp). Therefore, if APE1547 has the activity of FAE, it will shows perspective application in energy industry together with other enzymes to hydrolyze plant cell wall. The molecular docking of the substrates to activite site of APE1547 was performed using InsightII software. The results showed that the wild-type APE1547 had strong intermolecular interactions with the monoglycoester and diglycoester ferulate, and thus higher affinity. In addition, using AutoDock 4.0, APE1547 had lower free energy of binding for monoglycoeste and diglycoester ferulate. Therefore, we presumed that APE1547 had the hydrolytic activity similar to the FAE.

The hydrolysis of plant cell wall including ferulic acid was catalyzed by the wild-type APE1547 associated with other enzymes, and the yield of ferulic acid was low. To obtain the FAE of higher activity, DNA shuffling of APE1547 was employed. The large number of gene mutant library and the high-throughout screening method has been constructed. Through screening, we obtained the mutant AS610, which had high activity for hydrolyzing ferulic acid ethyl ester. Now, improving the hydrolysis efficiency of plant cell wall to produce ferulic acid and the enzymatic activity towards ferulic acid ethyl ester via directed evolution of the hyperthermophilic esterase APE1547 is still underway in our laboratory.

Addresses of participants

Mrs. Livnat J. Afriat
Weizmann Institute of Science
Faculty of Biochemistry
Department of Biological Chemistry
Herzl street 1
76100 Rehovot
Israel
livnat.afriat@weizmann.ac.il

Dr. Amir Aharoni
Ben Gurion University in the Negev
Department of Life Science
Ben gurion Blvd. 1
84105 Beer-Sheva
Israel
aaharoni@bgu.ac.il

Dr. Isabelle Andre
Laboratoire d'Ingénierie des Systèmes
Biologiques et des Procédés, INSA
Biocatalysis group
135, avenue de Rangueil
31077 Toulouse
France
isabelle.andre@insa-toulouse.fr

Mr. Mohamed Awadelkarim
University College Dublin
Conway Institute
School of Biomolecular
and Biomedical Science
Belfield
Dublin 4
Ireland
mohamed.awadelkarim@ucd.ie

Mrs. Hagit Bar
Weizmann Institute of Science
Faculty of Life Science
Department of Biological Chemistry
Ullman building, room 206
Herzl street 1
76100 Rehovot
Israel
hagit.bar@weizmann.ac.il

Dr. Paul Alan Bates
Cancer Research UK,
London Research Institute
Biomolecular Modelling Laboratory
44 Lincoln's Inn Fields
WC2A 3PX London
United Kingdom
paul.bates@cancer.org.uk

Dr. Nina Baudendistel
BASF SE
GVF/E, A030
Carl-Bosch-Str. 38
67056 Ludwigshafen
Germany
nina.baudendistel@basf.com

Prof. Stephen Benkovic
The Pennsylvania State University
Department of Chemistry
414 Wartik Laboratory
16802 University Park, PA
United States
sjb1@psu.edu

Mr. Baris Binay
Istanbul Technical University
Science and Letter Faculty
Department of Molecular Biology
and Genetics
Maslak
34469 Istanbul
Turkey
binay@itu.edu.tr

Prof. Uwe Bornscheuer
Greifswald University
Institute of Biochemistry
Department of Biotechnology
& Enzyme Catalysis
Felix-Hausdorff-Str. 4
17487 Greifswald
Germany
bornsche@uni-greifswald.de

Mr. Michael Breuer
BASF SE
Fine Chemicals & Biocatalysis Research
GVF/E - A030
67056 Ludwigshafen
Germany
michael.breuer@basf.com

Mr. Jan Brezovsky
Masaryk University
Faculty of Science
Loschmidt Laboratories
Kamenice 5/A4
625 00 Brno
Czech Republic
briza@chemi.muni.cz

Dr. Vânia Brissos
Universidade Nova de Lisboa
Instituto de Teconologia Química e Biológica
Department of Microbial & Enzyme
Technology
ITQB-UNL, Av. República, Estação
Agronómica Nacional
2780-157 Oeiras
Portugal
vbrissos@itqb.unl.pt

Dr. Janusz M. Bujnicki
International Institute of Molecular
and Cell Biology in Warsaw
Laboratory of Bioinformatics and Protein
Engineering
Molecular Evolution Group
ul. ks. Trojdena 4
02-109 Warsaw
Poland
iamb@genesilico.pl

Mrs. Maren Butz
ETH Zurich
Department of Chemistry and Applied Biosciences
Wolfgang-Paulistrasse 10
8093 Zurich
Switzerland
butz@org.chem.ethz.ch

Dr. Jose Daniel Carballeira Rodriguez
Instituto de Biomedicina y Biotecnología de Cantabria (IBBTEC)
Cardenal Herrera Oria s/n
39011 Santander
Spain
jdcarballeira@telefonica.net

Ms. An Cerdobbel
Ghent University
Faculty of Bioscience Engineering
Department of Biochemical and Microbial Technology
Coupure Links 653
9000 Gent
Belgium
an.cerdobbel@ugent.be

Prof. Anju Chadha
IIT Madras
Department of Biotechnology
Rm 404
600036 Chennai
India
anjuc@iitm.ac.in

Ms. Radka Chaloupkova
Masaryk University
Faculty of Science
Loschmidt Laboratories
Kamenice 5/A4
62500 Brno
Czech Republic
radka@chemi.muni.cz

Dr. Sihong Chen
University College Dublin
Conway Institute
School of Biomolecular and Biomedical Science
Belfield 4
Dublin
Ireland
sihong.chen@ucd.ie

Dr. Ghermes Chilov
MolTech, Ltd.
Leninskie gory, 1/75A
119992 Moscow
Russian Federation
ghermes@moltech.ru

Ms. Eva Chovancova
Masaryk University
Faculty of Science
Loschmidt Laboratories
Kamenice 5/A4
625 00 Brno
Czech Republic
akllupe@chemi.muni.cz

Dr. Fred Christians
Pacific Biosciences
1505 Adams Dr
94025 Menlo Park, CA
United States
fchristians@pacificbiosciences.com

Dr. Janine Copp
Victoria University of Wellington
School of Biological Sciences
PO Box 600
6140 Wellington
New Zealand
janine.copp@vuw.ac.nz

Dr. Marc Creus
University of Neuchatel
Faculty of Sciences
Department of Chemistry
Emile Argand 11, CP 158
CH-2009 Neuchatel
Switzerland
marc.creus@unine.ch

Prof. Jiri Damborsky
Masaryk University
Faculty of Science
Loschmidt Laboratories
Kamenice 5/A4
62500 Brno
Czech Republic
jiri@chemi.muni.cz

Mr. Maurycy Daroch
University of Liverpool
Faculty of Sciences
Department of Biological Sciences
Biosciences Building
Crown St
L69 7ZB Liverpool Merseyside
United Kingdom
m.daroch@liv.ac.uk

Dr. Tom Desmet
Ghent University
Faculty of Bioscience Engineering
Department of Biochemical and Microbial Technology
Coupure Links 653
9000 Gent
Belgium
t.desmet@ugent.be

Mrs. Edyta Dyguda-Kazimierowicz
Wroclaw University of Technology
Institute of Physical and Theoretical Chemistry
Wybrzeze Wyspianskiego 27
50-370 Wroclaw
Poland
edyta.dyguda@pwr.wroc.pl

Prof. Paul Engel
University College Dublin
Conway Institute, School of Biomolecular and Biomedical Science
Belfield
D4 Dublin
Ireland
paul.engel@ucd.ie

Prof. Yan Feng
Jilin University
Key Laboratory for Molecular Enzymology and Engineering
2519 Jiefang Road
130023 Changchun
China
yfeng@mail.jlu.edu.cn

Dr. Joshua Finkelstein
Nature
25 First Street
Suite 104
02139 Cambridge, MA
United States
j.finkelstein@boston.nature.com

Dr. Markus Fuhrmann
Sloning BioTechnology GmbH
Department of Genetic Diversity
Zeppelinstr. 4
82178 Puchheim
Germany
m.fuhrmann@sloning.com

Dr. Leonid Gaidukov
Weizmann Institute of Science
Department of Biological Chemistry
Ullmann Building
Herzl street
76100 Rehovot
Israel
leonid.gaydukov@weizmann.ac.il

Prof. John Gerlt
University of Illinois, Urbana-Champaign
Department of Biochemistry
419 Roger Adams Laboratory
600 South Mathews Avenue
61801 Urbana, IL
United States
j-gerlt@uiuc.edu

Dr. Michaela Edin
Royal Institute of Technology
School of Biotechnology
Department of Biochemistry
AlbaNova University Center
S-10691 Stockholm
Sweden
michaela.edin@biotech.kth.se

Dr. Steven Fairchild
The MITRE Corporation
Emerging Technologies Office
MS H205
7515 Colshire Drive
Mclean, Va 22102
United States
sfairchild@mitre.org

Mr. Valerio Ferrario
Università degli Studi di Trieste
Faculty of Pharmacy
Department of Pharmaceutical sciences
Piazza le Europa 1
34127 Trieste
Italy
vferrario@units.it

Dr. Toni Fleming
Piramal Healthcare
Department of Biocatalysis
P.O. Box 2, Belasis Avenue
Billingham
United Kingdom
Toni.Fleming@Piramal.com

Prof. Frederico Gago
Universidad de Alcalá
Facultat de Medicina
Departamento de Farmacaologia
Campus Universitario
Alcalá de Henares
28871 Madrid
Spain
federico.gago@uah.es

Dr. Pietro Gatti-Lafranconi
University of Milano-Bicocca
Deptartment of Science
Biotechnology and Biosciences
piazza della Scienza 2
20126 Milan
Italy
pietro.gattilafranconi@unimib.it

Mr. Lars Giger
ETH Zurich
Laboratory of Organic Chemistry
Department of Chemistry
Wolfgang-Pauli Strasse 10
HCI, F336
8093 Zurich
Switzerland
lars.giger@org.chem.ethz.ch

Mrs. Christiane Goedl
Graz University of Technology
Institute of Biotechnology
and Biochemical Engineering
Petersgasse 12/1
8010 Graz
Austria
christiane.goedl@tugraz.at

Dr. Catherine Goodman
Nature Chemical Biology
25 First Street
Suite 104
02141 Cambridge, MA
United States
c.goodman@boston.nature.com

Ms. Yosephine Gumulya
Max-Planck-Society
MPI of Coal Research
Kaiser-Wilhelm-Platz 1
45470 Mülheim An Der Ruhr
Germany
gumulya@mpi-muelheim.mpg.de

Dr. Volker Heinrichs
Athenix
Department of Directed Evolution
108 T. W. Alexander Drive
PO Box 110347
27709 Research Triangle Park, NC
United States
vheinrichs@athenixcorp.com

Dr. Claudia Hoebartner
Max-Planck-Society
MPI for Biophysical Chemistry
AG Nucleic Acid Chemistry
Am Fassberg 11
37077 Goettingen
Germany
claudia.hoebartner@mpibpc.mpg.de

Prof. Philipp Holliger
MRC Laboratory of Molecular Biology
Hills Road
CB2 2QH Cambridge
United Kingdom
ph1@mrc-lmb.cam.ac.uk

Dr. Olga Iranzo Casanova
Instituto de Tecnologia Química e Biológica
Estação Agronómica Nacional
Av. da República
2780-157 Oeiras
Portugal
oiranzo@itqb.unl.pt

Prof. Miguel González
University of Barcelona
Faculty of Chemistry
Department Química Física
C/ Martí i Franquès, 1
08028 Barcelona Catalonia
Spain
miguel.gonzalez@ub.edu

Dr. Daniela Grabs-Röthlisberger
University of Washington
Department of Biochemistry
Box 357350
98195 Seattle, WA
United States
roethlis@u.washington.edu

Prof. Dietmar Haltrich
University of Natural Resources
and Applied Life Sciences
Department of Food Sciences and Technology
Division of Food Biotechnology
Muthgasse 18
1190 Wien
Austria
dietmar.haltrich@boku.ac.at

Dr. Aurelio Hidalgo
Universidad Autónoma de Madrid
Faculty of Sciences
Centro de Biología Molecular “Severo Ochoa”
Nicolás Cabrera 1
Lab 109
28049 Madrid
Spain
ahidalgo@cbm.uam.es

Dr. Florian Hollfelder
University of Cambridge
School of Biology
Department of Biochemistry
80 Tennis Ct Rd
CB2 1GA Cambridge
United Kingdom
fh111@cam.ac.uk

Prof. Karl Hult
Royal Institute of Technology
Department of Biotechnology
Biocatalysis Group
Roslagstullsbacken 21
10691 Stockholm
Sweden
kalle@biotech.kth.se

Dr. Colin Jackson
CSIRO
Entomology
Clunies Ross Street
Black Mountain
2601 Canberra ACT
Australia
colin.jackson@csiro.au

Prof. Karl-Erich Jaeger
Heinrich-Heine-Universität Düsseldorf
Institut für Molekulare Enzymtechnologie
Forschungszentrum Jülich
52428 Jülich
Germany
karl-erich.jaeger@fz-juelich.de

Mrs. Andrea Jesenska
Masaryk University
Faculty of Science
Loschmidt Laboratories
Kamenice 5/A4
625 00 Brno
Czech Republic
andreaj@chemi.muni.cz

Mr. Aubrey Jones
Novozymes, Inc
1445 Drew Ave
95618, Davis, CA
United States
aujo@novozymes.com

Prof. Romas Kazlauskas
University of Minnesota
BioTechnology Institute
Gortner Avenue 1479
St. Paul, MN
United States
rjk@umn.edu

Mr. Martin Klvana
Masaryk University
Faculty of Science
Loschmidt Laboratories
Kamenice 5/A4
62500 Brno
Czech Republic
martink@chemi.muni.cz

Mrs. Lorena Knapic
Università Degli Studi di Trieste
Faculty of Pharmacy
Department of Pharmaceutical sciences
Piazzale Europa 1
34127 Trieste
Italy
lknapic@yahoo.com

Dr. Michael Kotik
Academy of Sciences of the Czech Republic
Institute of Microbiology
Videnska 1083
142 20 Prague
Czech Republic
kotik@biomed.cas.cz

Prof. Dick B. Janssen
University of Groningen
Groningen Biomolecular Sciences
and Biotechnology Institute
Biochemical Laboratory
Nijenborgh 4
9747 AG Groningen
The Netherlands
d.b.janssen@chem.rug.nl

Mr. Helge Jochens
University of Greifswald
Institute of Biochemistry
Biotechnology and enzyme catalysis
Felix-Hausdorff-Str. 4
17487 Greifswald
Germany
helge.jochens@uni-greifswald.de

Dr. Henk-Jan Joosten
Wageningen University and Research Centre
Faculty of Fungal Genomics
Department of Microbiology
Dreijenplein 10
6703HB Wageningen gelderland
The Netherlands
henk-jan.joosten@wur.nl

Ms. Olga Khersonsky
Weizmann Institute of Science
Department of Biological Chemistry
Ullmann building, Room 207
76100 Rehovot
Israel
olga.khersonsky@weizmann.ac.il

Dr. Jan Kmunicek
Masaryk University
Institute of Computer Science
Botanicka 68a
60200 Brno
Czech Republic
kmunicek@ics.muni.cz

Dr. Manfred Konrad
Max-Planck-Society
MPI for Biophysical Chemistry
Department of Enzyme Biochemistry
Am Fassberg 11
37077 Goettingen
Germany
mkonrad@gwdg.de

Mrs. Tana Koudelakova
Masaryk University
Faculty of Science
Loschmidt Laboratories
Kamenice 5/A4
62500 Brno
Czech Republic
tangerine@chemi.muni.cz

Dr. Marcin Krol
Cancer Research UK
Biomolecular Modelling Laboratory
44 Lincoln's Inn Fields
WC2A 3PX London
United Kingdom
marcin.krol@cancer.org.uk

Mr. Vincent Lafaquière
INSA Toulouse
Ingénierie des Systèmes Biologiques
et des Procédés
135 avenue de rangueil
31077 Toulouse
France
vincent.lafaquiere@insa-toulouse.fr

Dr. Thean Chor Leow
Universiti Putra Malaysia
Faculty of Biotechnology and Biomolecular Sciences
Department of Cell and Molecular Biology
UPM Serdang
43400 Serdang, Selangor
Malaysia
adamleow@biotech.upm.edu.my

Dr. Johanna Mansfeld
Martin-Luther University
Institute of Biochemistry and Biotechnology
Department of Biosciences
Kurt-Mothes-Straße 3
06120 Halle
Germany
mansfeld@biochemtech.uni-halle.de

Mr. Pavel Mazura
Mendel University of Agriculture and Forestry
Faculty of Agronomy
Department of Molecular Biology
and Radiobiology
Zemedelska 1
61300 Brno
Czech Republic
mazura@sci.muni.cz

Prof. Pierre Monsan
INSA University of Toulouse
Department of Biochemical Engineering
135 avenue de Rangueil
31077 Toulouse Cedex 4
France
pierre.monsan@insa-toulouse.fr

Dr. Adrian Mulholland
University of Bristol
School of Chemistry
Senate House
Tyndall Avenue
Bristol BS8 1TH
United Kingdom
adrian.mulholland@bris.ac.uk

Mrs. Julia Kufka
Leibniz Institute of Plant Biochemistry
Department of Bioorganic Chemistry
Weinberg 3
06120 Halle/saale Sachsen-Anhalt
Germany
wessjohann@ipb-halle.de

Mr. Christian Leitner
University of Natural Resources
and Applied Life Sciences
Department of Food Sciences
and Technology
Muthgasse 18
1190 Vienna
Austria
christian.leitner@boku.ac.at

Prof. Stephan Lutz
Emory University
Department of Chemistry
Emerson 205
1515 Dickey Drive
30322 Atlanta, GA
United States
sal2@emory.edu

Dr. Ligia Martins
Universidade Nova de Lisboa
Instituto de Tecnologia Quimica e Biologica
Microbial and Enzyme Technology Lab
Av da Republica
2780-157 Oeiras Oeiras
Portugal
lmartins@itqb.unl.pt

Dr. Vesna Mitchell
Codexis, Inc.
200 Penobscot Dr
94063 Redwood City, CA
United States
vesna.mitchell@codexis.com

Dr. Johannes Muellegger
Zymeworks Inc.
540 - 1385 West 8th Ave.
V6H 3V9 Vancouver, British Columbia
Canada
hannes@zymeworks.com

Ms. Nayla Munawar
University College Dublin
Conway Institute
School of Biomolecular and Biomedical Sciences
Belfield Campus
Dublin 4
Ireland
nayla.munawar@ucd.ie

Dr. Emily Mundorff
Codexis
200 Penobscot Dr.
94063 Redwood City, CA
United States
emily.mundorff@codexis.com

Mr. Rajesh Narayanan
University College Dublin
Conway Institute, School of Biomolecular and Biomedical Science
Department of Biochemistry
Belfield Campus
Dublin 4
Ireland
rajesh.narayanan@ucdconnect.ie

Dr. Wim Nerinckx
Ghent University
Department of Biochemistry, Physiology and Microbiology
K.L. Ledeganckstraat 35, 6th floor
9000 Ghent
Belgium
wimnerinckx@lavabit.com

Dr. Antje Neubauer
BioSilta Oy
Pentti Kaiteran Katu 1
P.O.Box 4300
90014 Oulu
Finland
polarcircle@gmx.de

Prof. Peter Neubauer
Technical University of Berlin
Straße des 17. Juni 135
10623 Berlin
Germany
peter.neubauer@oulu.fi

Dr. Adrian Ochoa-Leyva
National Autonomous University of México
Faculty of Biotechnology Institute
Department of Biocatlysis and Cell Engineering
Av. Universidad #2001, Col. Chamilpa
62210 Cuernavaca Morelos
Mexico
aol@ibt.unam.mx

Dr. Linda G. Otten
Delft University of Technology
Faculty of Biotechnology
Department of Biocatalysis & Organic Chemistry
Julianalaan 136
2628 BL Delft
The Netherlands
l.g.otten@tudelft.nl

Prof. Sven Panke
ETH Zurich
Institute of Process Engineering
Department of Mechanical and Process Engineering, Bioprocess Laboratory
Universitaetstrasse 6
8092 Zürich
Switzerland
panke@ipe.mavt.ethz.ch

Mr. Sebastian Pawlak
International Institute of Molecular and Cell Biology
Laboratory of Bioinformatics and Protein Engineering
Trojdena 4
02-109 Warsaw
Poland
sebastian@genesilico.pl

Prof. Gregory Petsko
Brandeis University
Chemistry Department
MS 015
415 South Street
Waltham MA 02454
United States
petsko@brandeis.edu

Prof. Juergen Pleiss
University of Stuttgart
Institute of Technical Biochemistry
Allmandring 31
70569 Stuttgart
Germany
juergen.pleiss@itb.uni-stuttgart.de

Dr. Zbynek Prokop
Masaryk University
Faculty of Science
Loschmidt Laboratories
Kamenice 5/A4
63700 Brno
Czech Republic
zbynek@chemi.muni.cz

Dr. Julie Rannes
University of Manchester
Manchester Interdisciplinary Biocentre
131 Princess street
M1 7DN Manchester
United Kingdom
julie.rannes@manchester.ac.uk

Prof. Manfred Reetz
Max-Planck-Society
MPI of Coal Research
Kaiser Wilhelm Platz 1
45470 Mülheim a.d. Ruhr
Germany
reetz@mpi-muelheim.mpg.de

Prof. Magali Remaud-Simeon
University de Toulouse
Institut National des Sciences Appliquées
Laboratoire Ingénierie des Systèmes
Biologiques et des Procédés
135, Avenue de Rangueil
31077 Toulouse
France
remaud@insa-toulouse.fr

Dr. Aelig Robin
Manchester University
Manchester Interdisciplinary Biocentre
131 Princess Street
M1 7DN Manchester
United Kingdom
aelig.robin@manchester.ac.uk

Dr. Andrew Sawayama
California Institute of Technology
Department of Chemistry and Chemical Engineering
1200 E California Blvd
91125 Pasadena, CA
United States
sawayama@cheme.caltech.edu

Prof. Manfred Schneider
Bergische Universität Wuppertal
FB C
Department Organische Chemie
Gaussstrasse 20
42097 Wuppertal
Germany
schneid@uni-wuppertal.de

Ms. Bettina Schreier
Max-Planck-Society
MPI for Developmental Biology
Spemannstr. 35
72076 Tuebingen
Germany
bettina.schreier@tuebingen.mpg.de

Dr. Melanie Schürmann
Codexis
Jülich Chiral Solutions GmbH
Prof.-Rehm-Str. 1
52428 Jülich
Germany
melanie.schuermann@codexis.com

Prof. Burckhard Seelig
University of Minnesota
Department of Biochemistry, Molecular Biology & Biophysics
140 Gortner Laboratory
1479 Gortner Avenue
55108 St. Paul, MN
United States
seelig@molbio.mgh.harvard.edu

Dr. Waander Riethorst
Sandoz GmbH
DMAI Biocatalysis
Biochemiestrasse 10
6250 Kundl
Austria
waander.riethorst@sandoz.com

Ms. Judith A. Sabaton
European Science Foundation
1, quai Lezay-Marnésia
BP 90015
F-67080 Strasbourg Cedex
France
judith.sabaton@orange.fr

Ms. Yolanda Schaerli
University of Cambridge
Department of Biochemistry
80 Tennis Court Road
Sanger Building
CB2 1GA Cambridge
United Kingdom
ys316@cam.ac.uk

Ms. Sarah Schneider
University of Stuttgart
Institute of Microbiology
Allmandring 31
70569 Stuttgart
Germany
imbsch@imb.uni-stuttgart.de

Dr. Martin Schuermann
DSM
Pharmaceutical Products / Innovative Synthesis & Catalysis
P.O. Box 18
NL-6160 MD Geleen
The Netherlands
martin.schurmann@dsm.com

Prof. Ulrich Schwaneberg
Jacobs University Bremen
School of Engineering and Science
Department of Biochemical Engineering
Campus Ring 1
28725 Bremen
Germany
u.schwaneberg@jacobs-university.de

Prof. Brian Shoichet
University of California, San Francisco
School of Pharmacy
Department of Pharmaceutical Chemistry
1700 4th Street
Byers Hall Room 508D
94158-2330, San Francisco, CA
United States
shoichet@cgl.ucsf.edu

Dr. Tatyana Shubina
Computer-Chemistry-Center
Nägelsbachstraße 25
91052 Erlangen
Germany
tatyana.shubina@chemie.uni-erlangen.de

Dr. Krzysztof Skowronek
International Institute of Molecular
and Cell Biology
Laboratory of Bioinformatics and
Protein Engineering
Trojdena 4
02-109 Warsaw
Poland
krzysiek@genesilico.pl

Dr. Radka Snajdrova
University of York
York Structural Biology Labortory
Department of Chemistry
Heslington
YO10 5YW York
United Kingdom
radka@ysbl.york.ac.uk

Prof. Patrice Soumillion
Université Catholique de Louvain
Institute of Life Sciences, Unité BIOC
Place Croix du Sud 4-5, box 3
1348 Louvain-la-Neuve
Belgium
patrice.soumillion@uclouvain.be

Dr. Jeffrey Lutje Spelberg
Codexis
Julich Chiral Solutions GmbH
prof. Rehmstrasse 1
52428 Julich
Germany
jeffrey.lutjespelberg@codexis.com

Mr. Oleg Stroganov
Moscow State University
Belozersky Institute of Physico-
chemical Biology
Department of Biokinetics
Khokhlova st., 6
119992 Moscow
Russian Federation
stroganov@belozersky.msu.ru

Mr. Dimitry Suplatov
Lomonosov Moscow State University
Faculty of Bioengineering and Bioinformatics
Leninskiye Gory, building 73
119992 Moscow
Russian Federation
genesup@belozersky.msu.ru

Dr. Remigijus Skirgaila
UAB ”Fermentas”
Graiciuno 8
02241 Vilnius
Lithuania
skirgaila@fermentas.lt

Dr. Thomas John Smith
Sheffield Hallam University
Biomedical Research Centre
Owen Building
Howard Street
S1 1WB Sheffield
United Kingdom
t.j.smith@shu.ac.uk

Dr. Pankaj Soni
Max-Planck-Society
MPI of Coal Research
Group Prof. Reetz
Kaiser-Wilhelm-Platz 1
45470 Mülheim An Der Ruhr
Germany
soni@mpi-muelheim.mpg.de

Mr. Oliver Spadiut
University of Natural Resources
and Applied Life Sciences
Division of Food Biotechnology
Department of Food Sciences and Technology
Muthgasse 18
1190 Vienna
Austria
oliver.spadiut@boku.ac.at

Dr. Michael Strerath
DIREVO Biotech AG
Nattermannallee 1
50829 Cologne
Germany
michael.strerath@gmail.com

Dr. Maria Suarez
Ecole Polytechnique
Laboratories Biochemie CNRS UMR7654
Route de Saclay
91128 Palaiseau Cedex
France
maria.suarez@polytechnique.edu

Prof. Vytas Svedas
Lomonosov Moscow State University
Faculty of Bioengineering
and Bioinformatics
Vorobjev Hills 1
119991 Moscow
Russian Federation
vytas@belozersky.msu.ru

Mr. Per-Olof Syrén
Royal Institute of Technology
Faculty of Biotechnology
Department Of Biochemistry
David Bagares Gata 6
111 38 Stockholm
Sweden
per-olof.syren@biotech.kth.se

Prof. Dan Tawfik
The Weizmann Institute of Science
Faculty of Biochemistry
Department of Biological Chemistry
76100 Rehovot
Israel
tawfik@weizmann.ac.il

Ms. Veena Thomas
University of California, San Francisco
Department of Pharmaceutical Chemistry
1700 4th Street, Room N508
Campus Box 2250
94158-2550 San Francisco, CA
United States
veenathomas@alum.mit.edu

Ms. Karolina L. Tkaczuk
IIMBC
Bioinformatics & Protein Engineering
ks.Trojdena 4
02109 Warsaw
Poland
poison-ivy@genesilico.pl

Dr. Sanja Tomic
University of Zagreb
Rudjer Boskovic Institute
Department of Physical Chemistry
Bijenicka 54
10000 Zagreb
Croatia
sanja.tomic@irb.hr

Ms. Maria Valentini
CRS4, Bioinformatica
Edificio 1, Parco Scientifico e Tecnologico
Sardegna Ricerche
09010 Pula
Italy
maria@crs4.it

Dr. Bert Van Loo
University of Cambridge
Department of Biochemistry
80 Tennis Court Road
CB2 1GA Cambridge
United Kingdom
bv223@mole.bio.cam.ac.uk

Mr. Mohamad Takwa
Royal Institute of Technology
Faculty of Biotechnology
Department of Biochemistry
Albanova university center
106 91 Stockholm
Sweden
takwa@biotech.kth.se

Mr. Tengku Haziyamin Tengku Abdul Hamid
University Putra Malaysia
Faculty of Biotechnology and Biomolecular Sciences
Department of Microbiology
43400 UPM Serdang, Selangor
Malaysia
haziyamin@iiu.edu.my

Dr. Janet Thornton
European Bioinformatics Institute
EMBL
Wellcome Trust Genome Campus
Hinxton
CB10 1SD Cambridge
United Kingdom
thornton@ebi.ac.uk

Dr. Nobuhiko Tokuriki
Weizmann Institute of Science
Department of Biological Science
76100 Rehovot
Israel
nobuhiko.tokuriki@weizmann.ac.il

Prof. Nicholas Turner
University of Manchester
Faculty of Engineering and Physical Sciences
Department of Chemistry
Manchester Interdisciplinary Biocentre
131 Princess Street
M1 7DN Manchester
United Kingdom
nicholas.turner@manchester.ac.uk

Dr. Jan Metske Van Der Laan
DSM Food Specialties
R&D/FTD/BND 699-0330
PO-box 1
NL2600MA Delft
The Netherlands
jan-metske.laan-van-der@dsm.com

Mr. Benoit Villiers
University of Cambridge
Department of Biochemistry
80 Tennis Court Road
CB21GA Cambridge
United Kingdom
bv228@cam.ac.uk

Dr. Rebecca C. Wade
EML Research
Villa Bosch Schloss
Wolfsbrunnenberg 33
69118 Heidelberg
Germany
rebecca.wade@eml-r.villa-bosch.de

Dr. Lenka Weignerova
Academy of Sciences of the Czech Republic
Institute of Microbiology
Center for Biocatalysis and Biotransformation
Videnska 1083
142020 Prague
Czech Republic
lenka@wcontact.cz

Dr. Montarop Yamabhai
Suranaree University of Technology
Faculty of Agricultural Technology
Department of Biotechnology
111 University Avenue
Meung district
30000 Nakhon Ratchasima
Thailand
montarop@sut.ac.th

Ms. Bo Yuan
The University of Manchester
School of Chemistry
Oxford Road
M13 9PL Manchester
United Kingdom
bo.yuan@postgrad.manchester.ac.uk

Dr. Guirong Zhang
Jilin University
College of Life Science
Qianjin Road 2699
130012 Changchun
China
zgr@jlu.edu.cn

Dr. Arieh Warshel
University of Southern California
Department of Chemistry
University Park
90089-1062 Los Angeles, CA
United States
warshel@usc.edu

Ms. Sarah Whitehead
Manchester University
Manchester Interdisciplinary Biocentre
131 Princess street
M1 7DN Manchester
United Kingdom
sarah.whitehead@postgrad.manchester.ac.uk

Mr. Emrah Yelboga
Istanbul Technical University
Faculty of Science and Letters
Department of Molecular Biology
and Genetics
Bolumu Maslak
34390 Istanbul
Turkey
yelboga@itu.edu.tr

Prof. Chul-Ho Yun
Chonnam National University
School of Biological Sciences
and Technology
300 Yongbong-dong, Buk-ku
500-757 Gwangju
Republic of Korea
chyun@jnu.ac.kr

Notes

Notes

ESF-EMBO Symposium

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Loschmidt Laboratories
Institute of Experimental Biology
Faculty of Science, Masaryk University
Brno, Czech Republic
http://loschmidt.chemi.muni.cz/peg/

Programme

Day 1 - October 25

Afternoon **Registration**

19.00 Welcome Drink

20.00 Supper

Day 2 - October 26

08.45 **Conference opening**

09.00 **Session 1**
Fundamentals of enzymatic catalysis

12.00 Lunch & coffee break

15.30 **Session 2**
Computer modelling in protein design

19.00 Dinner

20.00 **Poster Session I**
Authors with odd poster numbers

Day 3 - October 27

09.00 **Session 3**
Computer modelling in protein design

12.30 Lunch & coffee break

15.30 **Session 4**
Bioinformatics in protein design

19.00 Dinner

20.30 **Forward Look Plenary Discussion**

Day 4 - October 28

09.00 **Session 5**
Directed evolution of biocatalysts

12.30 Lunch

Afternoon **Half-day excursion**

19.00 Dinner

20.00 **Poster Session II**
Authors with even poster numbers

Day 5 - October 29

09.00 **Session 6**
Directed evolution and engineering of biocatalysts

12.30 Lunch & coffee break

15.30 **Session 7**
Directed evolution and engineering for biocatalysis

20.00 **Get-together & Conference Dinner**

Day 6 - October 30

Breakfast & Departure