THE DEVELOPMENT OF NEW BIOCATALYTIC REACTIONS FOR ORGANIC SYNTHEISS

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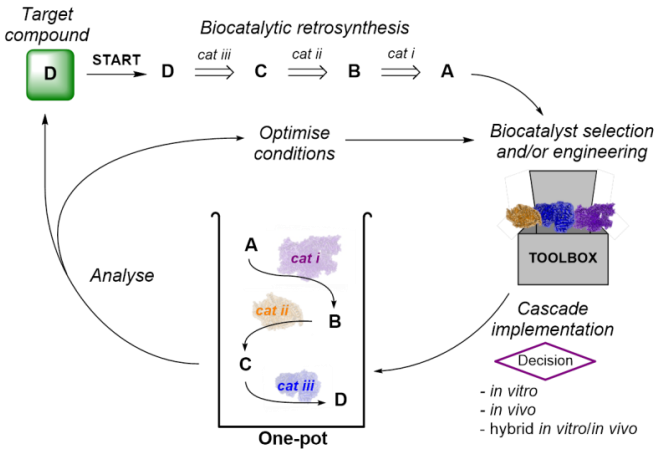
Key Words: enzyme discovery, cascade reactions, amines, directed evolution, protein engineering.

This lecture will describe recent work from our laboratory aimed at developing new biocatalysts for enantioselective organic synthesis, with emphasis on the design of in vitro and in vivo cascade processes for generating chiral pharmaceutical building blocks. By applying the principles of 'biocatalytic retrosynthesis' we have shown that it is increasingly possible to design new synthetic routes to target molecules in which biocatalysts are used in the key bond forming steps [1].



The integration of several biocatalytic transformations into multi-enzyme cascade systems, both in vitro and in vivo, will be addressed in the lecture. In this context monoamine oxidase (MAO-N) has been used in combination with other biocatalysts and chemocatalysts in order to complete a cascade of enzymatic reactions [2-4]. Other engineered biocatalysts that can be used in the context of cascade reactions include w-transaminases [5], ammonia lyases [6], amine dehydrogenases [7], imine reductases [8], and artificial transfer hydrogenases [9]. We shall also present recent work regarding the discovery of a new biocatalyst for enantioselective reductive amination and show how these enzymes can be used to carry out redox neutral amination of alcohols via 'hydrogen borrowing' [10].

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FUNCTIONAL DYNAMICS OF PROTEINS ON CATALYSIS FROM COMBINED QM/MM AND EXPERIMENTAL STUDIES

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STRUCTURAL INSIGHT INTO ENANTIOSELECTIVE INVERSION OF AN ALCOHOL DEHYDROGENASE REVEALS A "POLAR GATE" IN STEREO-RECOGNITION OF DIARYL KETONES

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Key Words: diaryl ketones; alcohol dehydrogenase; enantioselective inversion; (4-chlorophenyl)-(pyridine-2-yl)-ketone; polar gate

Diaryl ketones are generally regarded as "difficult-to-reduce" ketones due to the large steric hindrance of two bulky aromatic side-chains. Alcohol dehydrogenase from *Kluyveromyces polyspora* (*Kp*ADH) has been identified as a robust biocatalyst due to its high tolerance to diaryl ketone substrate (4-chlorophenyl)-(pyridine-2-yl)-ketone (CPMK), whereas with a moderate *R*-selectivity of 82% *ee*. To modulate the stereoselectivity of *Kp*ADH, a "polarity scanning" strategy was proposed, in which six key residues inside and at the entrance of substrate binding pocket were identified. After iterative combinatorial mutagenesis, variants Mu-R2 and Mu-S5 with enhanced (99.2% *ee*, *R*) and inverted (97.4% *ee*, *S*) stereoselectivity were obtained. Their crystal structures in complex with NADPH were resolved to elucidate the evolution of enantioselective inversion. Based on computational results from MD simulation, an approximate plane formed by α-carbon of four residues (N136, V161, C237 and G214) was identified at the entrance of substrate binding pocket of Mu-S5, which act as a "polar gate" for substrates. Due to the discrepancy in charge characteristics between chlorophenyl and pyridine substituents, pro-*S* orientation of CPMK is defined when it passing through the "polar gate" in Mu-S5, whereas the similar plane in WT is blocked by several aromatic residues. Our result represents advancement in engineering stereo-complementary ADH toward diaryl ketones, and provides structural insight into the mechanism of stereoselective inversion in *Kp*ADH.

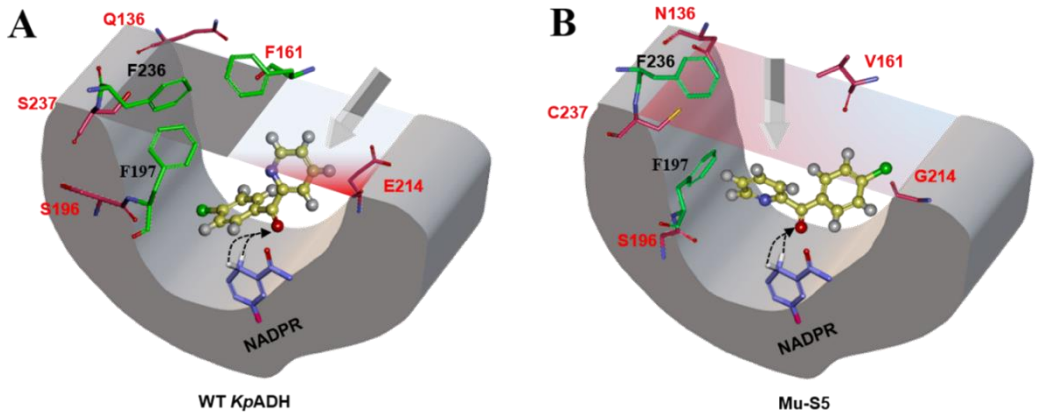

Figure 1 Typical catalytic conformations of (A) WT-CPMK$_{ProR}$ and (B) Mu-S5-CPMK$_{ProS}$ based on MD analysis; green sticks represent aromatic amino acids around CPMK; arrows indicate the entry direction of CPMK; the gray plane in (A) the entrance blocked by aromatic amino acids of WT KpADH; the blue and red planes in (B) the polar entrance of Mu-S5; purple sticks represent NADPH and black dashed lines indicate the direction of hydrogen attack; the mutation sites are marked in red.

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SURPASSING THERMODYNAMIC, KINETIC, AND STABILITY BARRIERS TO ISOMERIZATION CATALYSIS FOR TAGATOSE BIOSYNTHESIS

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Key Words: biocatalysis, conversion, rate, partitioning, equilibrium

D-Tagatose is a rare ketohexose sugar with sweetness similar to that of sucrose. However, its glycemic index and caloric value is much lower because of low bioavailability, making it an attractive GRAS (generally regarded as safe) sugar substitute. Recent studies have also demonstrated that it is anti-hyperglycemic and prebiotic, which promotes gut health. Thus, there exists a high demand in food industry for the economical production of rare sugars, like tagatose.

The enzyme L-arabinose isomerase (LAI) that responsible for the reversible isomerization of the pentose L-arabinose to L-ribulose can also isomerize the hexose D-galactose to D-tagatose. LAI has thus been the enzyme of choice to produce tagatose, although, to date, few commercial bioprocesses exist. A variety of LAIs from different microorganisms have been isolated and have reported optimal activity at a range of temperatures and pH. Some of the limitations of tagatose biosynthesis using LAI that may be hindering commercial viability are, 1) unfavorable enzymatic kinetics since galactose is not the native substrate of LAI, 2) low enzyme stability, particularly in the absence of divalent metal ions, and 3) low equilibrium constant for galactose to tagatose isomerization.

Few previous reports have been successful at engineering enzymatic properties of LAI for industrial application; often addressing only one of the bottlenecks to productivity. To address the kinetic issue, several groups have used enzyme engineering methods to enhance catalytic efficiency of LAI toward galactose and have shown moderate increases in productivity. To counter low-stability issues, many groups have tested the utility of thermophilic enzymes. However, most thermophilic enzymes rely on divalent metal ions (Mn^{2+}, Co^{2+}, Fe^{2+}) for stability, and high reaction temperatures (≥ 80 °C) result in significant caramelization, which are all undesirable and must be removed from product, adding to processing costs. Surface-display or encapsulation in particles or whole-cells can stabilize enzymes. Finally, the thermodynamic limitations of isomerization of galactose to tagatose are severe and, arguably, the most recalcitrant issue since $\Delta G^{\circ}_{rxn} \approx +1.2$ kcal/mol, which indicates theoretical maximum equilibrium conversion ~ 14 % at room temperature. Several approaches have been used to overcome this limitation. Thermophilic enzymes can achieve higher conversions than mesophilic enzymes since the equilibrium shifts toward tagatose at higher temperatures. Whole-cell biocatalysts with GRAS organisms (e.g. lactic acid bacteria (LAB) and *E. coli*) that disproportionately partition substrate and product across their membrane has also been shown to partially circumvent this thermodynamic limitation while simultaneously enhancing enzyme stability; albeit at a kinetic penalty imposed by substrate transport limitations. Recently, cell permeabilization and sugar transport overexpression were demonstrated as methods to overcome the kinetic penalty imposed by cellular encapsulation.

There have currently been no studies that look to systematically analyze all three limitations – kinetic, thermodynamic, and enzyme stability – of the enzymatic isomerization of galactose to tagatose. This work clearly demonstrates the presence of these three limitations and provides a novel approach to balance their advantages and limitations. We use the food-safe engineered probiotic bacterium Lactobacillus plantarum as the expression host due to its increasing relevance to biochemical and biomedical research. This approach enabled ~ 50 % conversion of galactose to tagatose in 4 h (productivity of ~ 38 mmol tagatose L^{-1} h^{-1}) ultimately reaching ~ 85 % conversion after 48 h at high galactose loading (300 mM) in batch culture. This is among the highest conversions and productivities reported to date for tagatose production using a mesophilic enzyme. Such an approach is expected to be applicable to other biocatalytic systems where similar trade-offs between kinetics, thermodynamics, and/or stability pose hurdles to process development.

This work is currently under consideration for publication:
Bober & Nair (2019) Nature Communications (in revision)
https://nature-research-under-consideration.nature.com/channels/1337-under-consideration/posts/44492-surpassing-thermodynamic-kinetic-and-stability-barriers-to-isomerization-catalysis-for-tagatose-biosynthesis

REVISITING ALCOHOL DEHYDROGENASES:
SELF-SUFFICIENT REGIO- AND ENANTIO-SELECTIVE FORMATION OF BI- AND TRI-CYCLIC LACTONES

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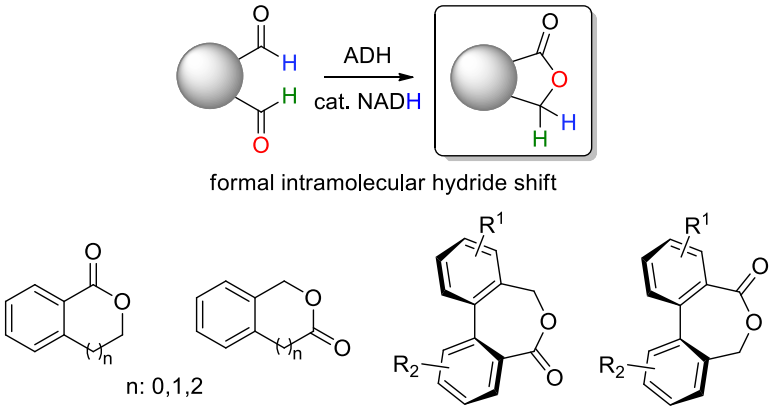
Key Words: Alcohol dehydrogenase, Bio-Tishchenko reaction, hydride shift, lactone, redox neutral.

The field of biocatalysis has witnessed over the past few years a renewed interest in the design of synthetic routes with high atom-economy, particularly of those employing redox enzymes. For long, the implementation of efficient recycling systems for cofactors (typically nicotinamide) essential to these enzymes was considered attractive for economical reasons, since the use of co-substrate and co-enzyme approaches [1] usually implied co-conversion of a cheap auxiliary substrate (*e.g.*, glucose, ethanol, acetone, formate). In line with growing awareness for sustainable technologies and the desire to reduce the environmental footprint of synthetic processes, strategies that bypass the need for stoichiometric amounts of co-subtrate or reagents are now being considered. For redox enzymes, this translates for instance into the enlargement of the scope of intramolecular hydride transfer, which allows biotransformations to run in closed loop. Exemplary are redox isomerization reactions applied to allylic alcohols or recently cyclic hydroxy-ketones [2]. In both cases, no new bond is created, and the hydride shift is responsible for a switch of functionality.
Owing to the dual redox reactivity of aldehydes and complementary activity of alcohol dehydrogenases (ADHs) on the aldehyde functionality (oxidation/reduction), a formal intramolecular biocatalytic hydride shift can be considered with dialdehyde molecules. Following our work on the disproporationation of aldehydes and the establishment of a biocatalytic Cannizzaro-type reaction using ADHs [3], we are now disclosing a broadly applicable enzymatic platform for the synthesis of bi- and tri-cyclic lactones starting from dialdehydes (Scheme 1). An intramolecular bio-Tishchenko reaction was developed with particular attention to redox economy. High turn-over numbers for the nicotinamide cofactor (up to 1.6×10^3 half-reactions) along with efficient 1,4-, 1,5- and 1,6-hydride shift on dialdehydes (1:1 ratio enzyme/cofactor) could be demonstrated, following reduction-oxidation sequence through lactol intermediate. Noteworthy, regio- and enantioselectivity were observed with a range of wild-type and engineered ADHs and preparative scale synthesis allowed isolation of several lactone products, with no concomitant waste generation [4]. Application of these lactones in (cross-)polymerization reactions is currently being investigated.



Scheme 1. Bio-Tishchenko reaction catalyzed by alcohol dehydrogenases and product scope

Acknowledgements
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EXPLORING MARINE CARBOHYDRATES: P450-CATALYZED DEMETHYLATION AND IDENTIFICATION OF A COMPLETE "PUL" FOR POLYSACCHARIDE DEGRADATION

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Key Words: Biocatalysis, CAZymes, Marine Carbohydrates, P450-Monooxygenase, Protein Engineering

Marine carbohydrates present in algal biomass are an emerging sustainable raw material for bioeconomy. The exploitation of algae as carbon source is hampered by our rather limited knowledge about the microbial pathways present in marine bacteria that can convert algal polysaccharides into oligo- and monosaccharides for fermentation into bioethanol or other compounds.

We have recently shown a distinct metabolic function of P450-monooxygenases in the degradation of agarose or porphyran, where the P450 enzymes (originating from *Formosa agariphila* or *Zobiella galactinovorans*) together with appropriate redox partners catalyze the demethylation of 6-*O*-methyl-D-galactose [1]. Furthermore, we have determined the crystal structure of the P450 enzyme and identified key residues essential for catalysis and substrate recognition [2].

More recently, we could elucidate the entire metabolic pathway involved in the degradation of a major cell wall polysaccharide using a set of enzymes present in the marine flavobacterium *Formosa agariphila* in a distinct and so far unexplored polysaccharide utilization locus (PUL). The pathway consists of 12 carbohydrate-active enzymes, including lyases, sulfatases and glycoside hydrolases that sequentially break down the complex polysaccharide into fermentable monosaccharides. For all previously unknown enzymes we performed a detailed biochemical characterization, determined several crystal structures and could identify the structures of all oligosaccharide intermediates formed during the complex enzymatic degradation by NMR spectroscopy and MS analysis [3].

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MULTI-ENGINEERING OF MICROBIAL CYTOCHROME P450 ENZYMES

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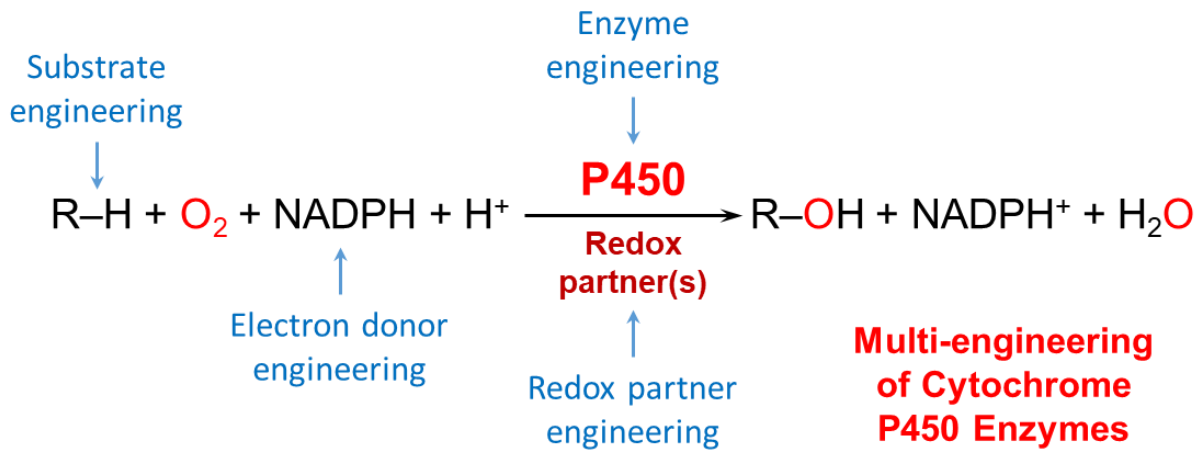
Key Words: Cytochrome P450 enzyme, Enzyme engineering, Substrate engineering, Redox partner engineering, Electron donor engineering

Selective oxidation of unactivated C–H bonds remains a central challenge in synthetic chemistry. Cytochrome P450 enzymes, a superfamily of ubiquitous hemoproteins, represent the nature's primary solutions to overcome this challenge. As promising biocatalysts for practical applications in pharmaceutical, biotechnological and chemical industries, P450 enzymes have attracted a wealth of attention due to their great versatility in catalyzing diverse oxidative reactions (*e.g.*, the sp^3 C–H hydroxylation and the sp^2 C=C epoxidation) on structurally complex and heavily functionalized substrates in regio- and/or stereoselective manners. However, wild type P450 enzymes usually show suboptimal activity, low stability, and narrow substrate spectra, which have significantly limited their broader applications. A typical P450 reaction system includes a P450 enzyme as the central catalyst, a substrate to be oxidized, redox partner proteins for electron transfer, NAD(P)H as the electron donor, and O_2 as the oxidant. In the past five years, we have made significant progresses on enzyme engineering, substrate engineering, redox partner engineering, and electron donor engineering for a number of microbial P450 enzymes. These multi-engineering efforts have generated useful engineered P450 catalytic systems for bio-production of pharmaceuticals, chemical intermediates, and biofuel molecules.



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DISSECTING POLYUNSATURATED FATTY ACID SYNTHASES FOR PRODUCT PROFILE CONTROL

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Key Words: polyunsaturated fatty acid synthase, polyketide, carbon chain length, cis-double bond, regulation

Polyunsaturated fatty acids (PUFAs) such as docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), and arachidonic acid (ARA) are essential fatty acids for humans and are ingested from fish oils. Because of increasing demand, however, fermentative processes using microalgae, yeasts, and fungi have been developed to produce DHA, EPA, and ARA, respectively. PUFAs are biosynthesized by either desaturases/elongases from oleic acid or PUFA synthases from acetyl units. PUFA synthases are composed of three to four subunits and each create a specific PUFA without undesirable byproducts even though the multiple catalytic domains in each huge subunit are very similar. In this study, we carefully dissected these PUFA synthases by in vivo and in vitro experiments and elucidated how the enzymes control PUFA profiles (Figure 1) [1]. Moreover, for the first time, we converted a practical microalgal DHA synthase into an EPA synthase based on the obtained results [2].

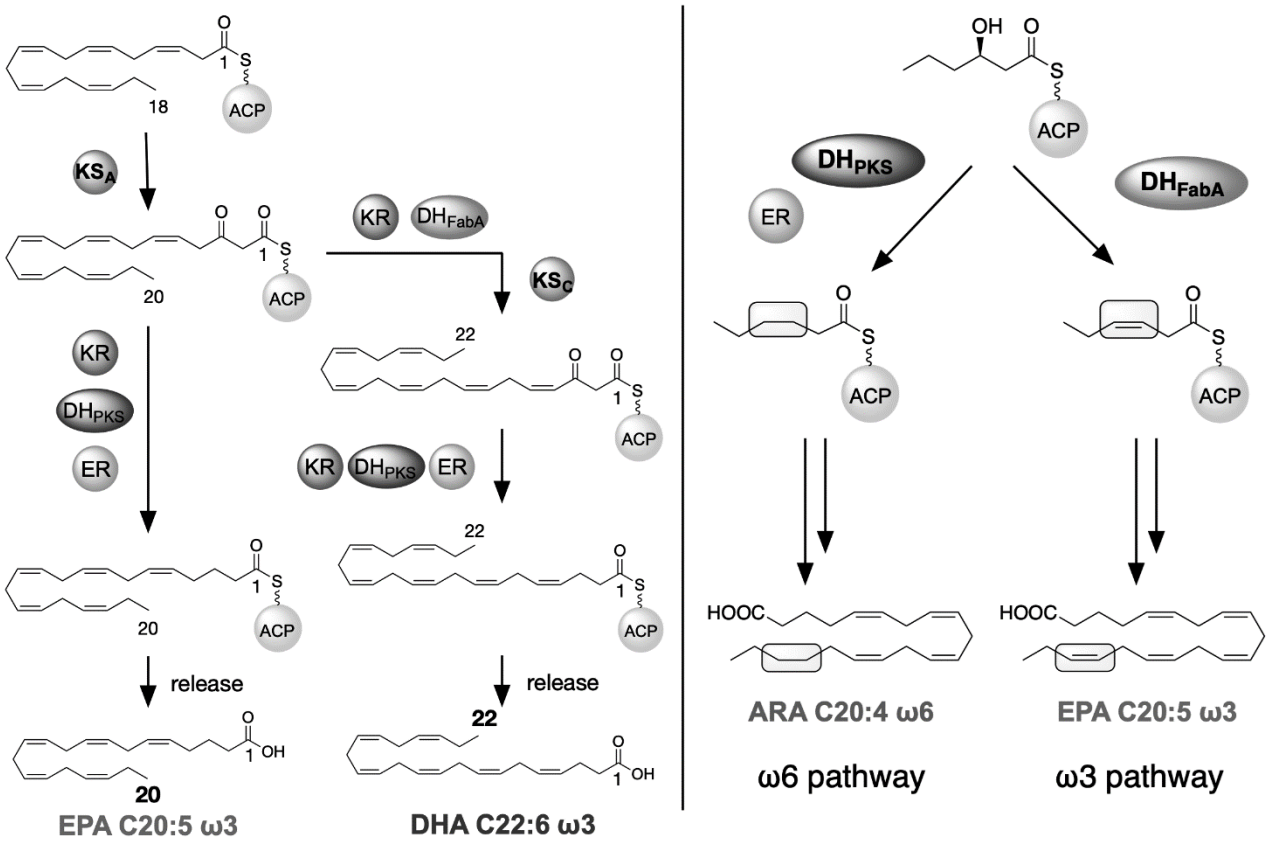

Figure 1 Control mechanism for carbon chain length (left) and first cis double bond formation (right) in PUFA synthases.

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PROMISCUITY, SERENDIPITY AND METABOLIC INNOVATION

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Keywords: promiscuity, evolution, metabolic pathway, mutation

Bioinformatic evidence suggests that metabolic pathways have evolved by “patchwork” recruitment of enzymes that have a promiscuous ability to catalyze a newly important reaction. However, we cannot explain why certain pathways arose rather than the thousands of other possibilities. Further, we have little insight into the *process* by which novel pathways were patched together and flux was improved via mutations.

We are studying the assembly of a novel pathway using a strain of *E. coli* in which *pdxB*, a gene required for synthesis of the cofactor pyridoxal 5’-phosphate (PLP), has been deleted. Because this strain cannot synthesize PLP, it cannot grow on glucose as a sole carbon source. We have evolved several lineages of the Δ*pdxB* strain in 0.4% glucose that can grow robustly on glucose. Each evolved strain has acquired 4-6 mutations. We have identified a four-step pathway patched together from promiscuous enzymes that bypasses the break in the PLP synthesis pathway caused by loss of PdxB (Fig. 1). We have identified the mechanisms by which three of the most common mutations in the adapted strains improve growth. We have also evolved several lineages of the Δ*pdxB* strain in 0.2% glucose. These strains show a strikingly different pattern of mutations, suggesting that they have either evolved a different mechanism to compensate for loss of PdxB, or have arrived at the same solution via a different evolutionary trajectory.

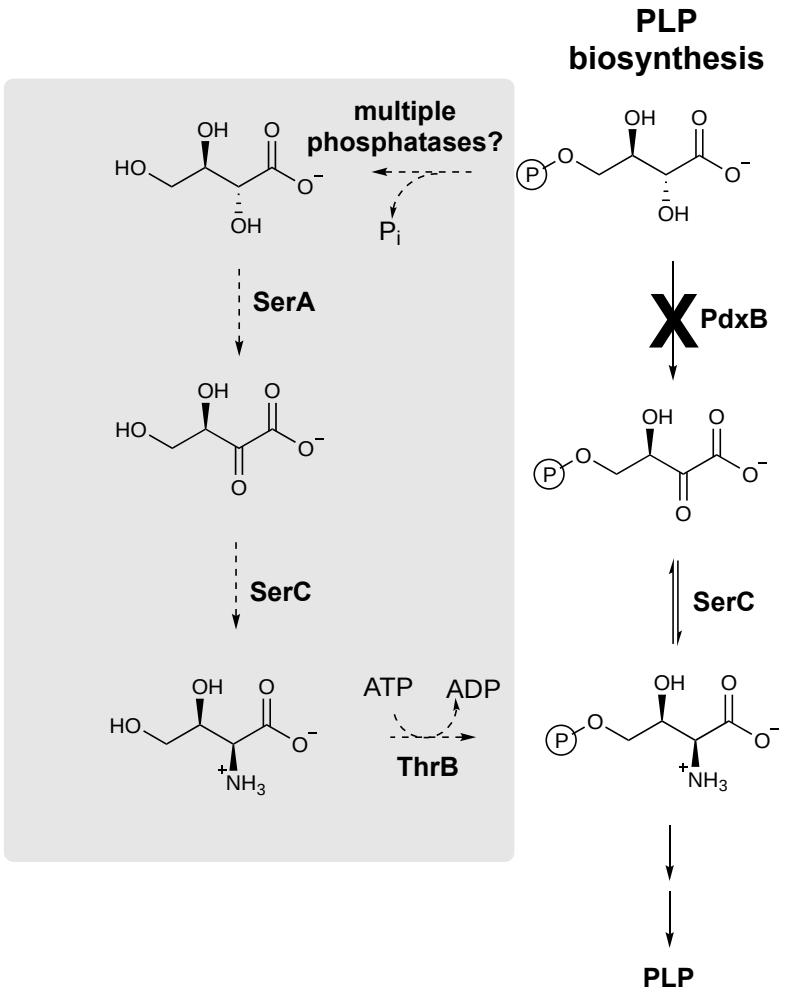

Fig. 1. A four-step pathway patched together from promiscuous activities of enzymes that normally serve other functions can bypass the break in PLP synthesis caused by loss of PdxB.

WHERE DO WE FIND NEW ENZYMES? - RULES AND TOOLS

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Enzymes for a variety of useful applications are required, but currently not known. Functional metagenomics and directed evolution promise access to such new catalysts, but the chances of finding them is low. Therefore high-throughput technologies are crucial to fight the odds. We have implemented workflows that allow screening of >10e6 clones and permit successful selections in picoliter water-in–oil emulsion droplets produced in microfluidic devices. While potentially faster, the vastness of sequence space (and the scarcity of 'solutions' in it) require strategies and rules for the identification and interconversion of enzymes. In this context 'promiscuous' enzymes have prominent roles: these catalysts possess additional activities in addition to their native ones, challenging the textbook adage "one enzyme – one activity", yet facilitating 'on-the-spot' utility for newly encountered challenges and also making future evolutionary transitions easier. Their molecular and mechanistic understanding may help us thus to chart routes towards new catalysts. Recent results of directed evolution, mechanistic investigation by kinetic and structural analysis and technology development are discussed in this context.

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STRATEGIES AND SOFTWARE FOR ENGINEERING PROTEIN TUNNELS AND DYNAMICAL GATES

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Key Words: Binding, Dynamics, Channels, Software, Strategy, Tunnels

Improvements in the catalytic activity, substrate specificity or enantioselectivity of enzymes are traditionally achieved by modification of enzymes' active sites. We have recently proposed that the enzyme engineering endeavors should target both the active sites and the access tunnels/channels [1,2]. Using the model enzymes haloalkane dehalogenases, we have demonstrated that engineering of access tunnels provides enzymes with significantly improved catalytic properties [3] and stability [4]. User-friendly software tools Caver [5], Caver Analyst [6], CaverDock [7] and Caver Web [8], have been developed for the computational design of protein tunnels/channels; FireProt [9] and HotSpot Wizard [10] for automated design of stabilizing mutations and smart libraries. Using these tools we were able to introduce a new tunnel to a protein structure and tweak its conformational dynamics. This engineering strategy has led to improved catalytic efficiency [2], enhanced promiscuity or even a functional switch (unpublished). Our concepts and software tools are widely applicable to various enzymes with known structures and buried active sites.

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EXPLORING SEQUENCE-FUNCTION SPACE IN THE OLD YELLOW ENZYME SUPERFAMILY

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Key Words: biocatalysis, ene-reductases, flavoenzymes, superfamilies, structure-function relationship

Biotechnology and bioinformatics have made it increasingly apparent that there is a vast wealth of protein 'dark matter', i.e., sequence and functional information that is yet to be discovered and harnessed for fundamental or applied gains. For example, the superfamily of Old Yellow Enzymes (OYEs) with ~88 characterized enzymes in the literature, is shockingly underexplored, despite >85 years of research and their proven industrial application. We have applied large scale bioinformatic and synthetic biology approaches to systematically sample and functionally characterize >120 representatives across the entire OYE superfamily, which is comprised of >70,000 members. Our efforts have more than doubled the current OYE knowledgebase and have yielded native biocatalysts with improved activity and expanded substrate specificity. Furthermore, our multidisciplinary approach serves as an adaptable pipeline for the analysis of other superfamilies, improving the current standard of investigative processes for the field. The comprehensive characterization of enzyme superfamilies, especially those with proven biocatalysis capabilities, offers tremendous opportunities for future developments of green and sustainable chemical processes.

UNDERSTANDING PROMISCUITY IN CLASS II PYRUVATE ALDOLASES

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Key Words: aldolase; promiscuity; pyruvate; hydroxypyruvate; phosphate activation.

Aldolases are grouped based on their strict donor specificity. To expand the use of these versatile enzymes it is essential to identify aldolases that utilize different donors. Recently a number of pyruvate aldolases that accepted hydroxypyruvate was identified [1]. The class II hydroxy ketoacid aldolase A5VH82 from *Sphingomonas wittichii* RW1 (*Sw*HKA) accepts hydroxypyruvate and was expressed for detailed studies. A thorough investigation including X-ray crystallography allowed new insights into the mechanistic details. This revealed a phosphate dependence of the enzyme and a preference for Mn (II) [2]. More importantly the substrate scope was studied. *Sw*HKA is promiscuous for the donor, it accepted pyruvate, hydroxypyruvate and the halopyruvates as donor molecules. This expands the range of products that can be synthesized with aldolases. In particular since the halogen containing products can easily be further modified by S_N2 reactions. Additionally, the substituted pyruvates yield products with not just one but two new stereocentres. Based on mutational studies, supported by structural elucidation by X-ray crystallography the stereochemical results will be discussed. Moreover, the acceptor molecules range far beyond the common sugar-type acceptors found in much pyruvate dependent aldolase chemistry. Overall *Sw*HKA enables with its promiscuous properties a significant extension of the aldolase toolbox (Figure 1).

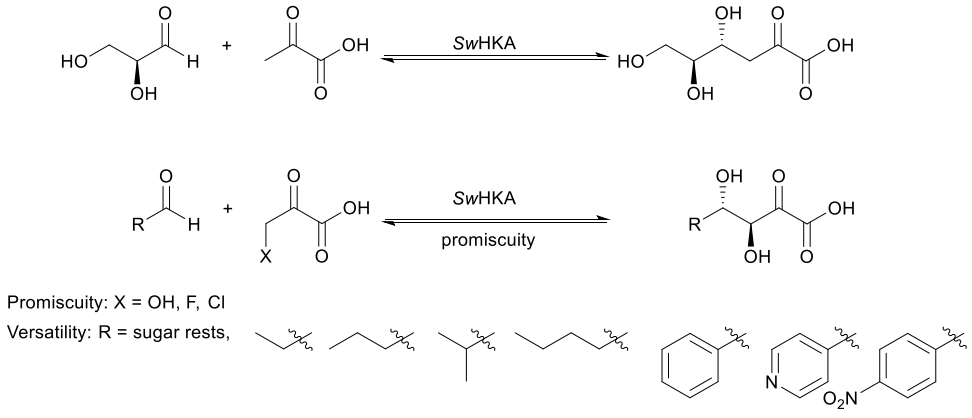

Figure 2 – SwHKA is a promiscuous aldolase that accepts a range of donors and acceptors.

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THRICE UPON A TIME: THE REPEATED EMERGENCE OF A NOVEL ENZYMATIC FUNCTION FROM AN EVOLVABLE PROTEIN SCAFFOLD

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Key words: functional diversity, sequence space, evolvability, nitroreduction.

Understanding the emergence of new protein functions from their ancestors is a long-standing challenge in biology and biotechnology; many questions remain unanswered. How can one protein scaffold support multiple distinct functions? How are diverse functions of a superfamily connected? How are major functional switches achieved? Large-scale experimental approaches that systematically determine the activity profiles across enzyme superfamilies have now begun to provide comprehensive views of functional diversity and evolutionary relationships. Intriguing insights can be gained: promiscuous activities are prevalent and many divergent proteins retain "functional connectivity" *via* enzyme promiscuity[1]. Interested in the varied biological and biotechnological roles of FMN-dependent “nitroreductase” enzymes (NTRs), we undertook extensive computational and functional analyses to determine sequence, structural and functional relationships[2]. This large and diverse superfamily contains >80,000 sequences from all domains of life, 54 structures, and >10 enzymatic functions. Our results suggest an evolutionary model in which contemporary subgroups of the superfamily have diverged in a radial manner from a highly “evolvable” minimal flavin-binding scaffold. To investigate the diverse NTR sequence space for the capacity to catalyze nitroreduction, we synthesized >500 genes and performed high-throughput activity screening to profile 18 *in vivo* substrates. *In vitro* kinetic analysis was subsequently performed on 112 enzymes against 32 substrates (*vs.* 2 nicotinamide cofactors), equating to >7,000 reactions[3]. We demonstrated that only four of the 22 distinct superfamily subgroups display canonical nitroaromatic reductase activities. Eight additional subgroups display occasional promiscuous activities with selected substrates, and 10 subgroups display no nitroreductase activity. Structural analyses revealed the underlying molecular details: nitroreduction has emerged three distinct times in the superfamily *via* three unique molecular solutions - loop insertions at three different positions in the NTR scaffold, combined with the fixation of key residues, have independently led to functional specialization. These results are now facilitating the rational redesign of the NTR scaffold. Our work provides clues for functional inference for sequences of unknown function, and will aid future efforts to exploit evolvable scaffolds for engineering, and understand the emergence of functional diversity in enzyme superfamilies.

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RECONSTRUCTION OF ANCESTRAL L-AMINO ACID OXIDASES TO BROADEN SUBSTRATE SELECTIVITY

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Key Words: ancestral sequence reconstruction, L-amino acid oxidase, L-arginine oxidase, substrate promiscuity

Characteristic functions of enzymes, such as high thermal stability and substrate specificity, are attained during the evolutionary process. Ancestral sequence reconstruction (ASR) is applied to infer the process by designing artificial enzymes which are located on ancestral node of phylogenetic tree; here, the inferred enzymes called ancestral enzymes. Ancestral enzymes often exhibit substrate promiscuity and high thermal stability of which functions are suitable to perform enzyme engineering. In addition, applicability of the ASR is high because the method requires only sequence data to design ancestral enzymes. Thus, we believe that artificial enzymes contributing to progress in enzyme engineering can be designed by ASR.

Success or failure of the ASR is strongly dependent on quality for multiple sequence alignment of sequence library which formed by sequences of target enzyme and their homologs. Thus, curation approach to generate optimal library is helpful to improve the succession rate. In this study, we tried to suggest the new approach through inferring of the evolutionary process of L-arginine oxidase (AROD), an FAD-dependent amino acid oxidase that exhibits high specificity toward L-arginine. Curation of AROD is difficult tasks because only one sequence data of native AROD is available for now; there is no structural and mutational data. Firstly, the library was prepared by selecting sequences that the 15th, 50th, 332nd and 580th residues are Gly, Ser, Trp and Thr, respectively. We omitted the sequences bearing extremely short or long and those with low sequence identity. The selection and exclusion of the sequences were performed by our original script. Finally, we can obtain three ancestral ARODs (AncARODn0, AncARODn1 and AncARODn2) using the library. In addition, we expressed the ancestral ARODs as well as native AROD (OkAROD) in bacteria. Phylogenetic tree analysis indicated that AncARODn0 is phylogenetically most remote from OkAROD whereas AncARODn2 is most similar to OkAROD (Figure 1).

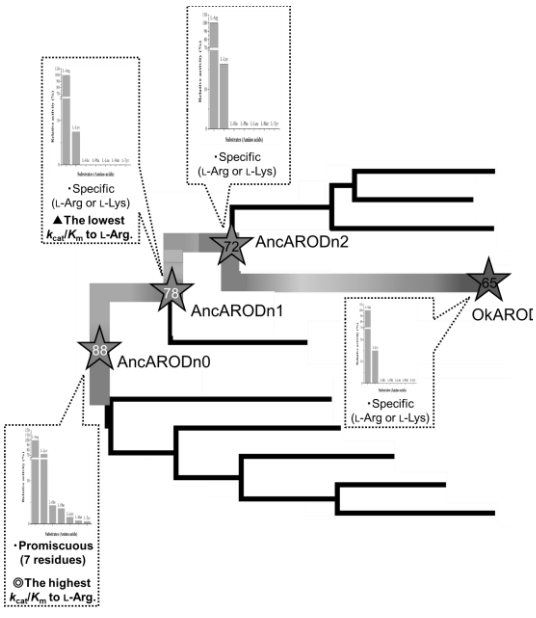

Figure 1. Schematic model representing biochemical properties of ancestral and native ARODs on a phylogenetic tree.

Biochemical analysis of the ARODs indicated that thermal stability was gradually increased by extending AROD sequences remote from native AROD. In fact, the $T_{1/2}$ values are following order: AncARODn0 (88 ºC) > AncARODn1 (78 ºC) > AncARODn2 (72 ºC) > OkAROD (65 ºC) (Figure 1). Remarkably, only AncARODn0 exhibits broad substrate selectivity similar to that of conventional promiscuous L-amino acid oxidase (LAAO) (Figure 1). Based on the results, we inferred that AROD may have evolved from a highly thermostable and promiscuous LAAO.

Furthermore, utilizing sequence data of AncARODn0 and identical procedure to design AncARODs, we succeeded in design of ancestral LAAO (AncLAAO) bearing broad substrate selectivity (> 10 of L-amino acids). AncLAAO can be expressed in soluble form utilizing BL21(DE3) expression system (>30 mg/L). AncLAAO can be used in deracemization of five DL-phenylalanine derivative with high enantio excess (> 99%, D-form) and conversion rate (> 76%). In this presentation, we will show the results of both AncAROD and AncLAAO.

THE COMING OF AGE OF DE NOVO PROTEIN DESIGN

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Proteins mediate the critical processes of life and beautifully solve the challenges faced during the evolution of modern organisms. Our goal is to design a new generation of proteins that address current day problems not faced during evolution. In contrast to traditional protein engineering efforts, which have focused on modifying naturally occurring proteins, we design new proteins from scratch based on Anfinsen's principle that proteins fold to their global free energy minimum. We compute amino acid sequences predicted to fold into proteins with new structures and functions, produce synthetic genes encoding these sequences, and characterize them experimentally. I will describe the de novo design of small molecule binding proteins and enzyme scaffolds, membrane penetrating macrocycles, transmembrane protein channels, and proteins that undergo conformational changes, including allosteric proteins that carry out logic operations.

MECHANISM, INHIBITION AND RECENT EVOLUTION OF AN UNUSUAL, PROMISCUOUS REDUCTASE

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Key Words: Dihydrofolate reductase, evolution of antibiotic resistance, enzyme inhibition

Dihydrofolate reductases (DHFR) catalyze the metabolically-essential reduction of dihydrofolate in an early step of purine biosynthesis. DHFRs are a central target in the control of proliferative diseases, including microbial infections. Trimethoprim is an effective antibiotic with broad clinical utility worldwide, acting as a selective inhibitor of microbial DHFRs. However, its clinical utility is threatened by the emergence of Type II microbial dihydrofolate reductases (DfrB) that are natively trimethoprim-resistant. DfrBs confer transmissible antibiotic resistance in humans and livestock. Their unusual structure and mode of substrate binding will be presented. We have created and screened active-site combinatorial libraries of a DfrB to provide key insights into the mechanism of this enzymatic reduction (1, 2). Attempts to identify promiscuous substrates for reduction have provided leads for inhibitor design. To this effect, we initiated a drug discovery program for these emerging targets. Fragment-based inhibitor development led to design of symmetrical bis-benzimidazoles that exhibit micromolar inhibition of DfrB1 (3). We determined that all closely-related Type II DHFRs share similar activity and inhibition patterns, broadening the utility of these inhibitors to the entire enzyme class. Upon whole-genome sequencing of trimethoprim-resistant *E. coli* from clinical isolates, we identified a *dfrB* gene flanked by multiple resistance genes, supporting its clinical emergence (4). To further investigate the evolutionary origin of this resistance enzyme, we undertook metagenomic screening. We have identified distantly-related structural homologs which, despite sharing only weak sequence homology, exhibit a high potential for conferring bacterial trimethoprim resistance.

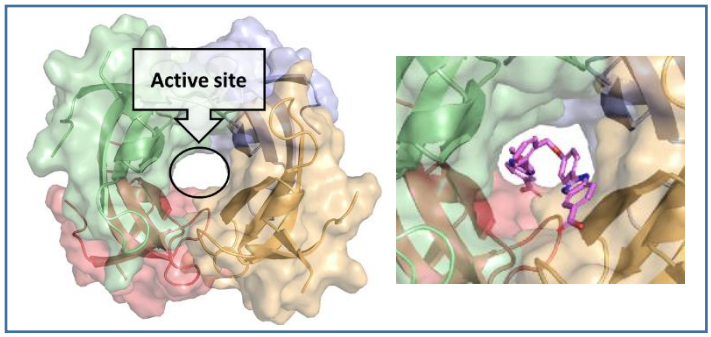

Figure 3 – The unique active site of the DfrB1 tetramer (left) is competitively inhibited with a designed bisbenzimidazole-type inhibitor (right).

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MASS-ACTIVATED SORTING OF NANOLITER DROPLETS FOR LABEL FREE SCREENING OF ENZYME LIBRARIES

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Directed Evolution is a key technology driving the utility of biocatalysis in pharmaceutical synthesis. Conventional approaches to Directed Evolution are conducted using bacterial cells expressing enzymes in microplates, with catalyzed reactions measured by HPLC, high-performance liquid chromatography-mass spectrometry (HPLC-MS), or optical detectors, which require either long cycle times or tailor-made substrates. To better fit modern, fast-paced process chemistry development where solutions are rapidly needed for new substrates, droplet microfluidics interfaced with electrospray ionization (ESI)-MS provides a label-free high-throughput screening platform. To apply this method to industrial enzyme screening and to explore potential approaches that may further improve the overall throughput, we optimized the existing droplet−MS methods. Carryover between droplets, traditionally a significant issue, was reduced to undetectable level by replacing the stainless steel ESI needle with a Teflon needle within a capillary electrophoresis (CE)−MS source. Throughput was improved to 3 Hz with a wide range of droplet sizes (10−50 nL) by tuning the sheath flow within the CE−MS source. The optimized method was demonstrated by screening reactions using two different transaminase libraries. Good correlations ($r2 \sim 0.95$) were found between the droplet−MS and LC−MS methods, with 100% match on hit variants. We further explored the capability of the system by performing in vitro transcription−translation inside the droplets and directly analyzing the intact reaction mixture droplets by MS. The synthesized protein attained comparable activity to the protein standard, and the complex samples appeared well tolerated by the MS. The success of the above applications indicates that the MS analysis of the microfluidic droplets is an available option for considerably accelerating the screening of enzyme evolution libraries.

COMPUTATIONAL ENZYME DESIGN FOR INDUSTRIALLY RELEVANT REACTIONS

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Key Words: computational enzyme design, asymmetric reactions, regioselectivity, *de novo* enzyme activity

Cognition of life from the perspective of synthetic biology centers on the design, construction, and characterization of novel biological systems using engineering design principles. Most catalytic functions in living organisms are achieved by enzymes, which serve as key components in synthetic biology. While the amino acid sequence makes up the primary structure of the protein, the chemical/biological properties of the protein are dependent on the three-dimensional or tertiary structure. In the nascent field of synthetic biology, researchers are striving to engineer novel biological functionality based on the structures. The development of computational enzyme design algorithms can provide large amounts of prototype molecules for the synthetic biological devices, especially for new catalytic devices, and also provide templates and guidelines for important components of synthetic biology. As the performance of computational algorithms and design strategies continue to improve, protein designers can construct enzymes from scratch and even create highly proficient biocatalysts. This talk provides a brief overview of design principles of proteins and highlights the latest examples of using computational methods to create enzymes for industrially viable applications.

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THE PEN CSR, USING EXTERNAL MOLECULAR PROGRAMS TO CONTROL DIRECTED EVOLUTION OF ENZYMES

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Key Words: Directed Evolution, Microfluidics, Molecular Programing, Self-Replication, Droplets

Selection-based strategies used in directed evolution methods proved to be very efficient but rely on the possibility of finding a case-specific selection tool that links the enzyme activity to its gene survival. Among all available directed evolution methods, the Compartmentalized Self-Replication (CSR) of Ghadessy and al. (1) and its derivatives allow to perform high-throughput in vitro selection tests thanks to microdroplets compartmentalization. Because engineered enzymes are often intended for use in unnatural environments, this kind of methods are particularly interesting. Yet, they can only be applied to polymerases replicating their own genetic sequence in the droplets.

Here, we used an external DNA-based artificial network to create a feedback loop linking the activity of a nicking enzyme to the replication of its own gene. Molecular networks, such as the one using the PEN DNA toolbox (2), are designed to produce short DNA strands interacting within each other's thanks to a set of enzymes. Taking short oligonucleotides at the input, they can generate short oligonucleotides of arbitrary sequence at the output. Enzyme activity can be assessed by these molecular networks thus allowing to produce a correlated amount of primers at the output. These primers can then be used to run the PCR of the enzyme's gene. Compared to the Compartmentalized Partnered Replication (CPR) (3), our method keeps the ability to perform in vitro assays of the target enzyme and does not necessitate any gene circuitry optimization.

Practically, bacteria carrying and expressing the mutants are co-encapsulated with the molecular program in individual droplets using microfluidics. The isothermal primers amplification (IPA) by the network is next initiated by raising the temperature to 45°C. After running the program for some time, each droplet contains an amount of the enzyme's gene forward and reverse primers corresponding to the activity of the enzyme expressed by the bacterium. A PCR is then launched in the droplets. Its yield in each droplet will then depend on the amount of primers, therefore on the enzyme activity. Up to 10^7 parallel self-selections can be run simultaneously on individual copy of the gene. After emulsion breakage, we retrieve a library enriched in the best mutant genes that can be used for a new cycle.

Two different selection pressures were applied by playing either on the IPA duration time to select for faster enzymes or by adding a heat shock at 65°C to select for higher thermostability. Next generation sequencing using MinION allowed us to follow the evolution process. This work is the first demonstration of the Programmable External Network based CSR (PEN CSR) method. Programs detecting other types of activity can be envisioned and would allow not only to greatly expand the scope of the CSR but also to implement smart selection functions.

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IPRO+/-: A COMPUTATIONAL PROTEIN DESIGN TOOL ALLOWING NOT ONLY FOR AMINO ACID CHANGES BUT ALSO INSERTIONS AND DELETIONS

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Key Words: protein, computational protein design, indels, mutation, optimization

The need for enzymes with new or improved catalytic properties and specificities underpins many challenges in both biotechnology and pharmaceutical industry. This is typically carried out by changing the native amino acid composition through single or multiple mutations or recombination. Many computational strategies have been developed for suggesting amino acid changes (i.e., mutations) likely to usher an altered substrate or cofactor specificity, improved thermostability or higher turnover. However, by perusing protein family alignments one can immediately notice the ubiquitous presence of gaps. These gaps imply that not all active enzyme variants have the same backbone length with insertions and deletions (indels) contributing significantly to the possibilities of altering enzyme activity by drastically affecting protein repacking. Currently, no algorithms exist which can systemically position multiple insertions or deletions during in silico protein redesign. In this contribution we introduce IPRO+/-, a first of its kind integrated environment for protein redesign with respect to a single or multiple binding imperatives by not only predicting amino acid changes, but also insertions and deletions (see Figure 1). IPRO+/- allows the user to run standalone programs for (a) predicting energy minimized structural models of an enzyme with a desired indels and/or mutations, (b) computing binding free energies between proteins and small molecules, and (c) performing energy minimization on any protein or protein complex. The contribution will provide an overview of the tasks involved in IPRO+/-, input language terminology, algorithmic details, software implementation specifics and application highlights. IPRO+/- will be made freely downloadable from http://www.maranasgroup.com/software.htm upon publication.

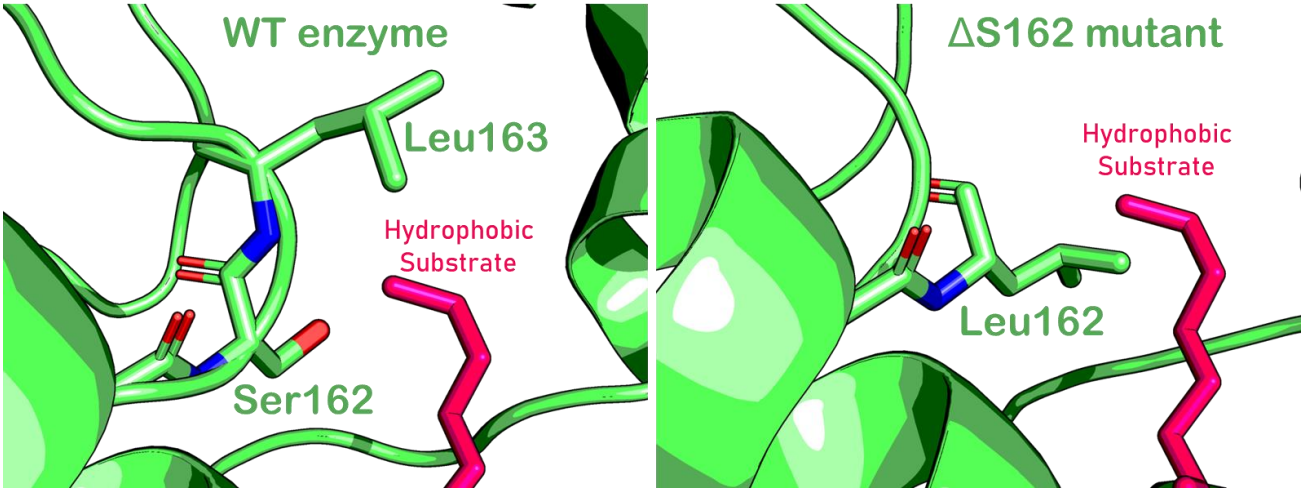

Figure 4. IPRO+/- designed enzyme which enhances binding to hydrophobic substrate by deleting the polar Ser162 that brings the Leu163 close to the omega carbon of the substrate.

7D QSAR BASED GRID MAPS GENERATED USING QUANTUM MECHANIC PROBES TO IDENTIFY HOTSPOTS AND PREDICT ACTIVITY OF MUTATED ENZYMES FOR ENZYME ENGINEERING

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Key Words: Enzyme engineering, Quantum Mechanics/Molecular Mechanics, Transition States, Grid map

Use of Quantum Mechanics hybridized with Molecular Mechanics (QM/MM) in Enzyme studies have greatly accelerated the finding of intermediate states of enzymatic reactions. The gaps in the conventional methods are in the identification of hot spots and screening enzyme variants. As a proof of concept, for the first time, receptor dependent – 4D Quantitative Structure Activity Relationship (RD-4D-QSAR) to predict kinetic properties of enzymes was demonstrated by Pravin Kumar et al, presented in Enzyme Engineering XXII, 2013, Toyama. We have extended this methodology to study enzymes using 7D-QSAR based grid maps. Induced-fit scenarios were explored using QM/MM simulations, which was then placed in a grid that stores interactions energies derived from QM parameters (QMgrid). The novelty of this method is that the mutated enzymes are immersed completely inside the QMgrid and this is combined with solvation models to predict descriptors; the grid captures the accurate electronic details of the reaction at very high resolution. Every grid point here is a probe, which are atoms that mimic atoms of the substrate interacting with the atoms of the enzyme, also atoms of the enzyme interacting with itself. The probes with its reaction coordinates are mapped on the ES complex conformations derived from ES, enzyme-transition and enzyme-product stages. The statistically relevant conformations are derived after screening using knowledge-based energy scoring matrices. The grid map shows high energy and low energy reactions across the ES system, which is used to pick hotspots. A substitution matrix is automatically constructed on the chosen hotspots using an evolutionary based scoring matrix coupled with statistical modelling process that gives the suited amino acids for a specific hot spot. We have tested this on a specific transaminase and QSAR models showed >90% specificity and >85% sensitivity towards the experimental activity with enzyme variants. Mapping descriptors on the enzyme structure revealed hotspots important to enhance the enantioselectivity of the enzyme. The method is efficient to design enzymes and proteins with minimum of double extending upto seven mutations on its own.

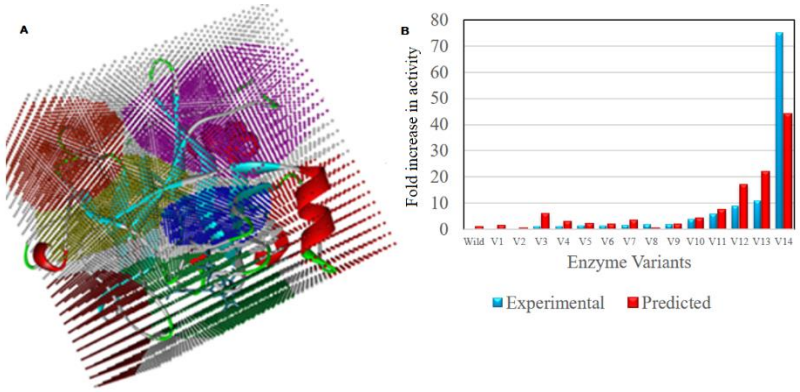

Figure 1A.QM grid where every gridpoint represents a probe. B. Comparative results of experimental and predicted activity of wild and variant transaminase

NEW TECHNOLOGIES FOR ENZYME ENGINEERING: COMBINING COMPUTATIONAL PREDICTIONS AND AUTOMATED EXPERIMENTAL FEEDBACK

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Key Words: simulation-experimental feedback loop, machine learning, fourth wave of enzyme engineering

The targeted design and optimization of novel enzymes and enzymatic reaction cascades increasingly demands a close connection between rational design, computational prediction and experimental feedback. In recent years, lots of effort have been put on increasing the throughput of experimental results, however, this approach frequently tends to stick in local minima and unsatisfying performance improvement despite considerable screening efforts. Contrary, model-based computational predictions, despite increasing available computation power, need to introduce severe simplifications and therefore will continue to lack accuracy and perfect predictability in the foreseeable future. The interplay of thorough model-based understanding, automated experimental feedback and, based on the latter, refinement of model predictions using for example machine learning methods, will in the near future become an important approach to combine the best of the two worlds. Ultimately, this provides potential to boost highly efficient automated or semi-automated design of new enzymatic properties in the scope of a “fourth wave” of enzyme engineering.

We present a new integrated directed evolution framework to achieve this simulation-experimental feedback loop, called “Feedback Guided Enzyme Optimization” (FEO). The implementation includes the setup of a suitable simulation back-end, robot-based experimental generation of mutants and evaluation of their performance [1], and finally feedback to the simulation in order to close the loop and verify and refine the quality of the predictions.Focus is laid on thorough statistical analysis of both prediction and experimental results, in order to tune false positive vs. false negative error rate, depending on experimental conditions: This includes, e.g., availability of time, ingredients, parallel workflows and distortions (random noise and potential systematic deviations) in both experimental and simulation setups.

The framework is being implemented in an automated robotic setup. We demonstrate results on three exemplary enzymatic systems: Firstly, GFP is employed as a simple role model to demonstrate the looping principle. The second example, aspartokinase III (AK3), is a key enzyme for the biosynthetic production of amino acids and derivatives thereof. Its activity is naturally limited by its own downstream products, e.g., lysine. Simulated predictions of the sensitivity of AK3 towards lysine have been compared to experimental data. This allowed a significant ($p<0.05$) simulation-based discrimination of highly resistant versus non-resistant variants. Determination of new lysine resistant mutants by multiple point mutations is performed within few dozen of iterations. The obtained candidates were validated, showing that new Lys-resistant variants can be obtained using the new workflow without special a priori knowledge or extensive (random) screening.

The third and most sophisticated enzyme system is the pyruvate dehydrogenase complex (PDC) which involves interesting features like shielding of reaction intermediates, renewal of co-factors, self-assembly, modularity and others. Based on recently published models of PDC by our group [2-3] and in collaborations [4], we demonstrate how the dynamic self-assembly of mutants of PDC and structurally similar enzymes complexes can be predicted, iteratively refined and in the future used for the creation of new enzyme cascades.

This presented framework is expected to have large impact on design and evolution of novel biomolecules and biosystems.

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IN SILICO ENZYME ENGINEERING – THE IMPORTANCE OF FAST AND ACCURATE ALGORITHMS

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Key Words: enzyme engineering, molecular modeling, in silico mutagenesis, computational design

Computer simulations are already widely used to rationally engineer new enzymes with improved properties. But if we can accurately screen millions of enzyme variants in a computer, then we can move into a new generation of *in silico* enzyme evolution. At ZYMVOL we are to be able to produce, model and rank protein-substrate interactions (including full protein dynamics) for over 50.000 enzyme variants per day. We have accelerated physics-based simulations, and combined experimental data with quantum approaches to develop a highly predictive computational platform. Our ZYMEVOLVER software can effectively reduce experimental validation to a few hundred variants and enzyme optimization campaigns to less than 6 months.

We will illustrate how we are creating custom-made enzymes for industrial applications.

NEW ENGINEERED PEPTIDE LIGASES AND SUBSTRATE PHAGE LIBRARIES FOR UNDERSTANDING CELLULAR PROTEOLYSIS

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Proteolysis is one of nature's major post-translational modifications and we need robust technologies to identify natural substrates and specificities for proteases. We have designed new peptide ligases capable of ligating peptide tags onto the N-terminus of newly proteolyzed proteins. These ligases allow identification of native protease substrates and cleavage sites both inside and outside of cells. We have also designed new substrate phage libraries coupled with deep sequencing to allow characterization of protease specificity. I'll present these new technologies that we believe are general to characterizing protease clients and specificity.

RECONSTRUCTION OF THE ACTIVE SITE OF A BACTERIAL PHOSPHOTRIESTERASE FOR THE CATALYTIC HYDROLYSIS AND DETOXIFICATION OF ORGANOPHOSPHATE NERVE AGENTS

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Key Words: phosphotriesterase, organophosphate nerve agents, detoxification

The bacterial phosphotriesterase (PTE), originally purified from the bacterium *Pseudomonas diminuta*, catalyzes the hydrolysis of the organophosphate insecticide paraoxon at a rate near the diffusion-controlled limit. The protein has been crystallized and the three-dimensional structure determined to high resolution. The protein adopts a $(\beta/\alpha)_8$-barrel structural fold and the active site is dominated by a binuclear metal center with a bridging hydroxide that is used for direct nucleophilic attack on the phosphorus center of the substrate. The wild-type enzyme is stereoselective for the hydrolysis of chiral organophosphate substrates. For example, the wild-type enzyme preferentially hydrolyzes the S_P-enantiomer of methyl phenyl *p*-nitrophenyl phosphate by a factor of 93:1. The mutation of Gly-60 to alanine (G60A) enhances the preference for hydrolysis of the S_P-enantiomer to a factor of 13,000:1 by reducing the rate of hydrolysis of the R_P-enantiomer. The stereoselectivity of PTE can be reversed by mutation of three residues within the active site (I106G/F132G/H257Y) and now the R_P-enantiomer is preferentially hydrolyzed by a factor of 118:1. Therefore, the stereoselectivity can be changed by more than four orders-of-magnitude by mutation of only four amino acids changes in the active site and thus PTE variants can be used to resolve racemic mixtures of chiral organophosphate esters. PTE was demonstrated to catalyze the hydrolysis of the organophosphate nerve agents tabun (GA), sarin (GB), soman (GD), cyclosarin (GF), VX, and VR. Screening of a small library of active site mutants identified one mutant (H257Y/L303T) that was particularly efficient toward the hydrolysis of the G-agents (in collaboration with Dr. Steve Harvey). The YT mutant of PTE hydrolyzed GB with a k_{cat} of 3.1×10^4 min^{-1}. This mutant, at a dose of 1 mg/Kg, was shown to significantly protect guinea pigs from exposure to sarin. The LD_{50} increased by more than a factor of 65 (in collaboration with Dr. Douglas Cerasoli). Coating of the surface of the YT variant with a thin ultrahydrophilic semipermeable poly(carboxybetaine) polymer enhanced protein stability, reduced immunological complications and protected rodents from repeated exposures to sarin over a period of 1-week (in collaboration with Dr. Shaoyi Jiang). From a 30,000 member mutant library, multiple variants were identified for the catalytic hydrolysis of VX and VR. The best mutants identified to date for the hydrolysis of (S_P)-VX and (S_P)-VR were enhanced more than 10,000-fold, relative to the wild-type PTE (in collaboration with Dr. S. Harvey). These results demonstrate that the active site of PTE can be readily manipulated to engineer protein variants with enhanced catalytic properties for hydrolysis of highly toxic organophosphate nerve agents.

VALIDATION AND STABILIZATION OF A PROPHAGE LYSIN OF *CLOSTRIDIUM PERFRINGENS* BY YEAST SURFACE DISPLAY AND CO-EVOLUTIONARY MODELS

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Key Words: lysin, antimicrobial proteins, protein engineering, co-evolutionary models, homolog

Bacteriophage lysins, which enzymatically degrade peptidoglycans within the cell wall of bacteria, exhibit high specificity and activity towards host bacteria with which the phage co-evolved. These properties of lysins make them attractive for use as antimicrobials. Though there has been significant effort to develop platforms for rapid lysin engineering, there have been numerous shortcomings when pursuing the ultra-high throughput necessary for discovery of rare combinations of mutations to improve performance. In particular, the biotechnological utility and evolvability of lysins would be aided by elevated stability. Lysin catalytic domains, which evolved as modular entities distinct from cell wall binding domains, can be classified into one of several families with highly conserved structure and function, many of which contain thousands of annotated homologous sequences. Motivated by the quality of this evolutionary data, the performance of generative protein models incorporating co-evolutionary information was analyzed to predict the stability of variants in a collection of 9,749 multi-mutants across 10 libraries diversified at different regions of a putative lysin from a prophage region of a *Clostridium perfringens* genome. Protein stability was assessed via a yeast surface display assay with accompanying high-throughput sequencing. Statistical fitness of mutant sequences, derived from second-order Potts models inferred with different levels of sequence homolog information, was predictive of experimental stability with AUCs ranging from 0.78 to 0.85. To extract an experimentally derived model of stability, a logistic model with site-wise score contributions was regressed on the collection of multi-mutants. This achieved a cross-validated classification performance of 0.95. Using this experimentally derived model, 5 designs incorporating 5 or 6 mutations from multiple libraries were constructed. All designs retained enzymatic activity with 4 of 5 increasing melting temperature, with the highest performing design achieving an improvement of +4 °C. In addition to validation of a putative lysin and stabilization thereof, the experimental and computational methods presented herein offer a new avenue for improving protein stability and is easily scalable to analysis of tens of millions of mutations in single experiments.

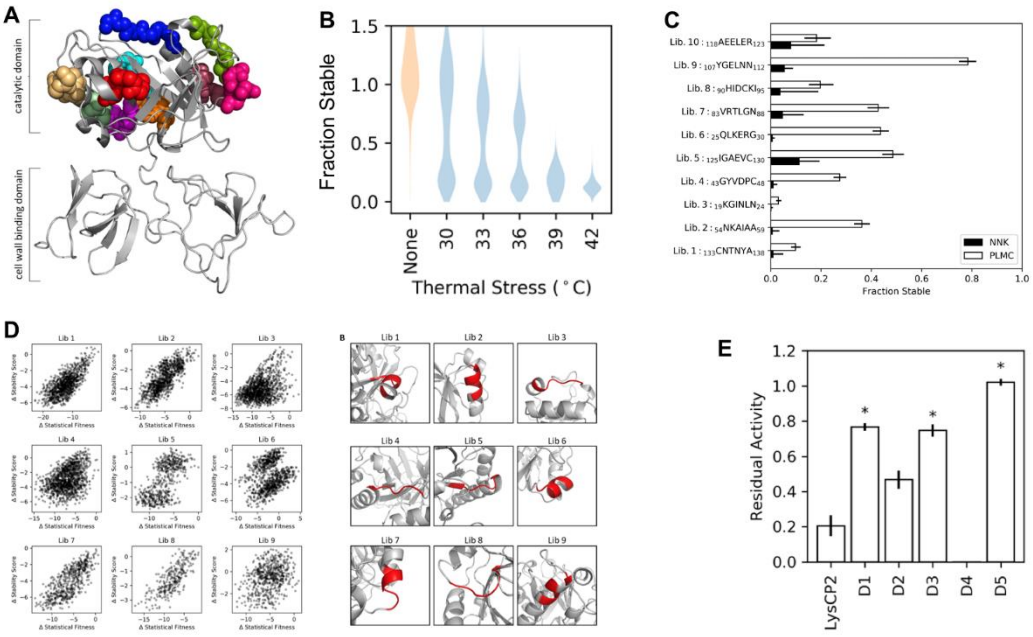

Figure 5. (A) Bacteriophage lysin LysCP2 homology model with diversified sites highlighted. (B) LysCP2 libraries exhibit a broad distribution of stability; high and low stability variants were selected by flow cytometry. (C) The co-evolutionary model yielded more stable lysin variants than a random control library. (D) The predictive quality of the experimentally-derived mutant model was strong at select locations (sub-libraries). (E) Second-generation designed mutants exhibit substantially improved activity after thermal stress relative to wild-type.

HIGH-LEVEL EXPRESSION, HIGH-THROUGHPUT SCREENING AND DIRECT RECOVERY OF NITROREDUCTASE ENZYMES FROM METAGENOME LIBRARIES

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Key Words: New tools; biomedical applications; enzyme promiscuity; metagenome screening

We have developed generally applicable library generation methods to maximize expression of cloned environmental genes, enabling screening for weak phenotypes in metagenome libraries. Our method also permits direct recovery of the encoded enzymes, providing rapid access to an almost unlimited diversity of previously unexplored biocatalysts. We have exemplified this for nitroreductases, members of a diverse family of oxidoreductase enzymes that can catalyze the bioreductive activation of nitroaromatic prodrugs such as metronidazole. These capabilities have diverse applications in medicine and research, including anti-cancer gene therapy and targeted ablation of nitroreductase-expressing tissues in transgenic animal models. However, research in these fields has largely been focused on the canonical nitroreductase NfsB from *Escherichia coli*, which exhibits sub-optimal levels of metronidazole activity. In previous work we have investigated alternative nitroreductase enzymes, sourced from genome-sequenced bacteria. To complement this work we have now turned to the discovery of novel nitroreductases from metagenomic DNA fragments, derived from the uncultivable bacteria present in New Zealand soil and lichen species.

ENZYMATIC TRANSFORMATION OF ANTIBODIES TO OBTAIN SINGLE GLYCOFORMS

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Introduction: Antibodies are synthesized in mammalian cell culture as heterogeneous mixtures of glycoforms. Therefore the glycan profile of monoclonal antibodies harvested from a bioreactor consists of various glycans depending upon the culture media, the control parameters and the metabolic status of the cells. Isolation of single glycoforms remains a challenge despite their value as agents of specific biological function. We report a method of sequential enzymatic-based changes to antibodies while trapped on an affinity column following harvest from a bioreactor. This method of solid phase enzymatic transformation is superior to previously reported methods because it allows a series of enzymatic steps without the need for intermediate purification of the antibody.

Results: Antibodies (camelid, Cetuximab and polyclonal human) were isolated on a solid-phase lectin column and their glycans modified by a sequential addition of enzymes for a desired transformation. Galactosylated antibodies (>90% yield) were produced by a two stage reaction. Sialylated antibodies (>90%) were produced by a 3 stage reaction involving sialidase, galactosyltransferase and finally treatment with the 2,6 sialyltransferase in the presence of CMP-NANA. Equimolar quantities of monosialylated and disialylated forms of the human antibodies (150 kDa) were produced and the results suggest that further sialylation may be limited by steric constraints within the dimeric structure. The ability to sialylate the smaller camelid antibody (80 kDa) was much greater with a high (>90%) yield of disialylated glycan structures, indicating that the steric constraints were less.

Significance: The biological activity of an antibody is highly dependent upon the glycan structure, which is described as a critical quality attribute for clinical efficacy. It is well reported that non-fucosylated or low galactosylated antibodies have high antibody-dependent cellular cytoxicity (ADCC). Sialylated antibodies have significant activity as ant-inflammatory agents. Therefore, isolating a single glycoform targeted for a specific biological activity offers considerable advantages. The method described in this abstract of enzymatic transformation during antibody purification provides a relatively simple solution to obtaining single glycoforms without the need for complex control during bioprocessing.

NEW ENZYMES FOR CELL SURFACE MODIFICATION: TOWARDS UNIVERSAL BLOOD AND IMPROVED ORGAN TRANSPLANTS

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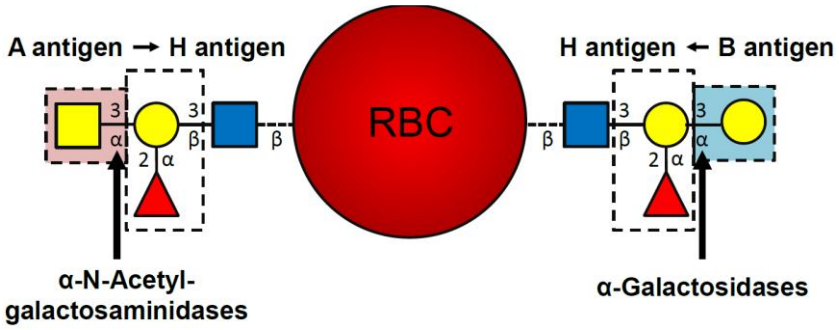
Key Words: Metagenomics, ABO blood system, glycosidases, antigen compatbility

Mammalian cell surfaces are coated in specific sugar structures, many of which function as antigens and are involved in cellular recognition. Important examples are the oligosaccharide A, B, and H antigens present on red blood cells that differentiate the A, B and O blood types. Enzymatic cleavage of the GalNAc and Gal residues from the cell surface would allow conversion of A and B red blood cells, respectively, to O type. Since Type O blood can be universally donated to patients with the same Rh factor, access to efficient enzymes would greatly broaden and simplify blood supply. We have sought such enzymes in metagenomic libraries derived from the human gut microbiome.



Total DNA was extracted from feces samples, fragmented into chunks containing ~30-40 genes (40-50 kB) and transformed into E. coli. After picking colonies into 384 well plates we screened them for enzymes that can be used to remove the Gal or GalNAc residues that function as the antigenic determinants from A and B type red blood cells, thereby generating “universal” O type blood. A set of efficient enzymes of a new class has been identified and characterised and used to convert whole units of A blood to O. These enzymes work approximately 30 times faster than any previously characterized and with high specificity. Further, they function well in whole blood thus can be hopefully integrated into the current blood processing process. Further we are exploring the potential of these enzymes for the removal of A and B antigens from organ surfaces prior to transplantation to reduce adverse immune responses. Attempts to engineer these enzymes for improved performance will also be presented.

A FACILE SCHEME FOR BIOSYNTHESIS OF PEPTIDES WITH NO LENGTH CONSTRAINTS

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Key Words: peptide synthesis, intein, enzyme evolution, self assembly peptides

While peptide drugs have become a viable class of biomedicines, efficient peptide expression and purification remains a critical technological need. We previous discovered that a number of self-assembling peptides such as 18A (EWLKAFYEKVLEKLKELF) and ELK16 (LELELKLKLELELKLK), when fused terminally to a target protein, can drive the target protein into active protein aggregates *in vivo*. A simple and rapid scheme for expression and purification of recombinant proteins using *Escherichia coli* was thus devised, by inserting a self-cleavable intein like Mxe GyrA between the self-assembling peptide and the target protein. In this scheme, the fusion protein is first expressed in the form of active aggregates, then separated by centrifugation upon cell lysis. Subsequently, the DTT-mediated intein self-cleavage reaction releases the target protein into solution. These self-assembling peptides together with the associated inteins constitute a set of cleavable self-aggregating tags (cSA), and provide an efficient route for the production of proteins with modest purity. More recently, this scheme has been applied to the biosynthesis of peptides, in particular those with lengths greater than 20 amino acids. A more efficient intein has also been engineered to afford the generation of authentic N-termini for the peptides. We believe this scheme will facilitate the development of more peptide drug candidates, and also lowering the costs of production of peptides of any length.

PROTEIN AND PROCESS ENGINEERING TOWARDS BIOCATALYSTS USEFUL IN INDUSTRIAL PROCESSES

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While progress is slower than anticipated, biocatalysts do enter an increasing number of proces-ses not just to pharmaceuticals but also to specialty and even bulk chemicals. The presentation will start with an update on some well-known and some less widely disseminated applications.

Next, we will cover two case studies from our own work, penicillin G acylase (Pen G acylase, PGA) and NADH oxidase (nox2). Both enzymes did and continue to benefit from protein engineering for optimum use in large-scale processes. Pen G acylase catalyzes amide bond formation to beta-lactam antibiotics, such as commercially important amoxicillin and cephalexin, but requires long time-on-stream as reaction yields are moderate and thus cost of goods are high. Development of continuous processes demands accurate process models [1-2], which in turn necessitates detailed knowledge about effects of immobilization and deactivation.[3]

NADH oxidase (nox2) finds use in biooxidation processes for the regeneration of NADH to NAD+.[4] However, the enzyme is sensitive to gas-liquid interfaces [5] and is turnover-limited owing to overoxidation at the active site.[6-8] We will report on progress to tackle such process stability issues.

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ENZYME ENGINEERING FOR INDUSTRIAL APPLICATIONS AT BASF

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BASF, a leader in performance chemicals, creates sustainable solutions for agricultural, food and industrial applications by harnessing the power of biotechnology. BASF's strong foundation of enzyme technology expertise spans over 20 years and includes the use of biocatalysts to manufacture chemicals as well as the development and sales of enzymes in animal feed, grain processing and personal care markets. In this presentation, we will discuss the role of rational and directed evolution enzyme engineering approaches for optimizing industrial enzymes for market applications.

ENGINEERING OF INDUSTRIAL BIOCATALYSTS

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Key Words: rational engineering; biocatalysts; PAMO; transaminase; industry.

In the last 40 years advances in the protein engineering have prompted the application of biocatalysts in the synthesis of building blocks, fine and bulk active pharmaceutical chemicals for the agrochemical, food, biofuel and pharmaceutical industries. Computational chemistry methodologies are fueling the development of a new generation of rationally designed biocatalysts with enhanced selectivity and specificity at a fraction of the time and cost compared to traditional protocols such as directed evolution.

We present two examples of rational enzyme design. Our first example is the study of the phenylacetone monooxygenase (PAMO), the most stable and thermo-tolerant member of the Baeyer–Villiger monooxygenases family. We solved the catalytic mechanism of this enzyme for the native substrate phenylacetone as well as for a linear non-native substrate 2-octanone, using molecular dynamics simulations, quantum mechanics and quantum mechanics/molecular mechanics calculations.1 By studying relevant PAMO variants we provide a theoretical basis for the preference of the enzyme for the native aromatic substrate over non-native linear substrates.2

The second example regards an (S)-selective-transaminase from Vibrio fluvialis (S-TAm), which offers an environmentally sustainable synthesis route for the production of pure chiral amines.3 By applying a rational enzyme engineering protocol we altered this enzyme towards better acceptance of bulky ketones, starting with no detectable activity of the WT. Our best S-TAm variant improved the reaction rate by > 1716-fold and retained activity even at 50 °C. To obtain such an outstanding result we only screened 113 variants, a substantially lower number than those typically associated with directed evolution (104 to 107 clones).
Both studies provide fundamental insights into the rational engineering of enzymes for industrial applications.

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IN SILICO ENGINEERED SmSDR ENZYME FOR THE PREPARATION OF ENANTIOPURE *R*-PHENYLEPHRINE

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Key Words: Protein engineering, Phenylephrine, QZyme Workbench™, HPMAE, Chiral, Enzyme engineering

It is a well-established fact that *in silico* enzyme engineering approaches such as molecular modeling, docking, simulation, provide molecular level understanding of the function of biocatalyst and specifically the role of mutational sites. These standalone protocols deliver the understanding of either Michaelis complex formation or the transition state formation or entry/exit path of the substrate. However, an intelligent workflow of these protocols can be applied for not only engineering of enzymes but to design them *in silico*. Quantumzyme has developed QZyme Workbench™, an integrated workflow using open source computational methods and proprietary scripts for scanning all the events involved in the biocatalytic process. The aim of this workbench is to conduct *in silico* protein design and is routinely leveraged to engineer enzyme for achieving desirable functions.

In the current study, we implemented our workbench to engineer a short chain dehydrogenase/reductase (SmSDR) enzyme to prepare *R*-Phenylephrine (*R*-PE) via stereoselective reduction of 1-(3-hydroxyphenyl)-2-(methylamino) ethanone. *R*-PE is one of the commonly used pharmaceutical analogs of Adrenalins. In order to avoid extreme conditions required for chemical methods, an optimal solution is to introduce an enzyme which exhibits high activity, and enantioselectivity to accomplish the asymmetric reduction step to yield enantiopure *R*-PE. Several such engineered enzymes are available for the synthesis of different biologically active compounds. In the present work, we have engineered a variant of SDRs enzyme to have high activity and enantio-specificity, focusing on the Michaelis complex formation using QM/MM method.

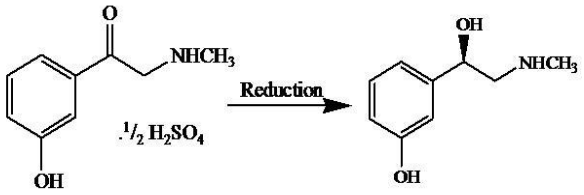

Figure 1 – Enzymatic reduction scheme of HPMAE to R-PE

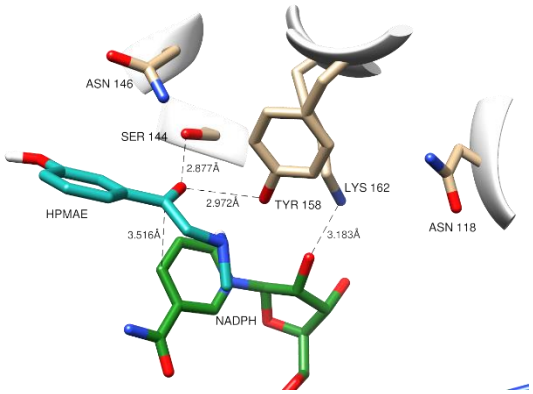

Figure 2 – Active site of SmSDR for the enzymatic reduction of HPMAE to R-PE

ADDRESSING THE PROBLEM OF PLASTIC WASTE: DEVELOPMENT OF AN ENZYMATIC PROCESS FOR PET RECYCLING

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Key Words: Polyethylene terephthalate, PET, waste recycling, industrial enzyme process, enzyme engineering

Every day, media and NGOs describe the society's disaffection for plastics accused of polluting the planet. All major brand-owners made commitments to solve this problem (e.g. Coca-Cola, Nestlé, Danone, PepsiCo, Suntory, Unilever, L'Oréal, Nike) and announced a future with less plastic waste by 2025.
Nevertheless, only 6 years before the announced term, no effective solution is yet available to meet these goals. Indeed, existing technologies like thermo-mechanical recycling leads to loss in mechanical properties of the polymer and even if several chemical recycling processes are under development, they suffer from the disadvantages of using organic solvents, high reaction temperatures and the need of an intensive waste sorting. Consequently, enzymatic recycling appears as a pertinent solution notably because the enzyme selectivity avoids a drastic sorting of waste and enables the recycling of complex plastics (multi-layers construction in some bottles of sparkling water for instance), it is an eco-friendly reaction in water and because of savings in energy consumption due to a low temperature of reaction.

Using a computer-aided engineering strategy, we drastically improved the depolymerizing performance of the best identified enzyme candidate. Utilizing site-directed mutagenesis targeted at the active site, combined with three-dimensional fold stabilization, we engineered an enzyme variant, demonstrating an astounding increase in thermostability combined with a high activity. This enzyme is able to depolymerize 90% of PET waste (200g/kg) into monomers, terephthalic acid and ethylene glycol, in less than 10 hours.

The downstream processing was developed and optimized leading to the demonstration that this enzymatic technology could enable the use of an industrial plastic waste to produce again PET monomers and ultimately a bottle from this recycled PET.

We hope to demonstrate the strong potential of the enzymatic technology jointly developed by CARBIOS and LISBP to provide a breakthrough solution to help solve society's growing plastic waste problem.

ENGINEERING ENZYMES TO CONTROL THE CHAIN-LENGTH SELECTIVITY OF BIOSYNTHESIZED OLEOCHEMICALS

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Key Words: Oleochemical, metabolic engineering, thioesterase, fatty acid, Escherichia coli.

Oleochemicals, a class of aliphatic molecules derived from lipids, are used in a range of applications including transportation fuels, consumer products (e.g. cosmetics, shampoo, cleaners), and industrial products (e.g. surface coatings, paints, lubricants, bioplastics). The most common oleochemicals are surfactants (e.g. sodium dodecyl sulfate) and biodiesel. Currently, the majority of oleochemicals are made from inexpensive lipid sources such as plant oils. Growing demand for oleochemicals, and in particular biodiesel, has led to an increased production of plant oil crops and raised concern about the sustainability and environmental impact of oil seed production. Consequently, interest in identifying alternative oleochemical feedstocks has grown. Many types of oleochemicals (e.g. free fatty acids, alcohols, methyl-ketones, olefins, alkanes, esters) have been produced in engineered microbes grown on a variety of carbon sources. That said, products made this way are not widely available. At least three major barriers remain — high feedstock costs, low yields, and a lack of selectivity towards desired molecules.

In this talk, I will discuss the development of enzymes capable of targeting the highly valued medium chain length products. I will describe pathways for producing high-value commodity chemicals derived from fatty-acids and how my group and others have combined synthetic biology and systems biology to improve oleochemical production in bacteria using sustainable feedstocks. I will highlight the use of heterologous plant and bacterial enzymes to alter the chain length distribution of products from common long-chain molecules to higher-value medium-chain analogs. I will describe bioprospecting, structure-guided mutagenesis, and directed evolution approaches that have successfully increased the selectivity and/or activity of enzymes to produce eight-carbon chain-length products. I will conclude with commentary on the remaining barriers to commercializing these technologies and areas where further research investment could prove fruitful.

HIGH-THROUGHPUT ENZYME ENGINEERING FOR COMMERCIAL-SCALE PRODUCTION OF NATURAL PRODUCTS

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The natural products world is unparalleled in its molecular diversity and wide application space. There are however numerous challenges associated with realizing the full potential of these molecules. Amyris has fermentatively produced eight different molecules at commercial scale. This track record is due to investment in advanced tools for strain engineering, high throughput screening, analytics, and bioinformatics. An integrated pipeline encompassing these tools has enabled Amyris to rapidly accelerate the engineering cycle and reduce the number of design-build-test iterations needed for microbial production of any natural product. In this presentation, we will discuss how this infrastructure is now being leveraged for high-throughput enzyme mutagenesis and screening, enabling greater access to natural products and their derivatives. Further, the application of our massive screening infrastructure to enzyme libraries would not be possible without equally sophisticated statistical models and data analysis tools. Scientists at Amyris are accessing ever greater portions of the enzyme sequence space to improve specificity and activity – ultimately enabling sustainable industrial-scale production of natural products. This talk will describe how each aspect of the enzyme engineering pipeline has led to rapid and high-quality screening of hundreds of thousands of mutants for multiple enzymes.

MULTIDIMENSIONAL ENGINEERING OF CHYMOSIN FOR EFFICIENT CHEESE PRODUCTION BY MACHINE-LEARNING GUIDED DIRECTED EVOLUTION

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Key Words: ProteinGPS, Multidimensional Engineering, Substrate Specificity, Design of Experiment

The global cheese market today exceeds $100B/year. Chymosin (a.k.a. rennin) is an aspartic endopeptidase produced by the stomach lining of new-born mammals. During cheese production chymosin is added to the milk where it cleaves the glycomacropeptide (GMP) from the surface of casein micelles to initiate milk coagulation. Current commercial recombinant chymosin enzymes derived from *Bos taurus* (cow) or *Camelus dromedarius* (camel) are limited in their proteolytic specificity leading to incomplete milk-to-cheese conversion. Increasing the chymosin specificity for GMP cleavage would significantly decrease the amount of milk needed for cheese production thereby reducing cost and decreasing environmental footprint of the dairy industry. Separate from milk coagulation, chymosin dependent release of N-terminal peptides from alphaS1 casein during cheese ripening leads to unwanted softening, accompanied with cheese loss during industrial processing such as slicing and shredding. Furthermore, chymosin dependent cleavage of the C-terminal end of beta casein contributes to unwanted bitterness of the cheese. Improvement of chymosin proteolytic specificity in both milk coagulation and cheese ripening is consequently of high commercial relevance.

We here present how we applied the ProteinGPS protein engineering platform to improve the specificity of camel chymosin for GMP cleavage, increase the milk clotting efficiency, while simultaneously reduce the off-target casein cleavage in cheese resulting in unwanted softening and bitter taste. A total of 108 amino acid substitutions found in naturally existing chymosin homologs or selected from structure-based design approaches were systematically introduced into the enzyme backbone using Design of Experiment principles for a total of ~300 chymosin variants distributed over four iterations (Fig. 1). The relative contribution and epistatic effect of each of the 108 substitutions in each functional dimension was modeled independently using modern machine learning algorithms. As little as 300 different chymosin variants covered the statistical sampling of a total sequence space of ~10^{32} illustrating the information efficiency of the method. This low number of chymosin variants to be produced and analyzed was small enough to allow for near-product grade quality in application relevant assays, including an LC-MS/MS-based mapping of casein degradation products in micro cheeses. The best performing engineered chymosin is 9 amino acid substitutions away from the parent camel chymosin, has increased GMP cleavage specificity by 30-fold, doubled milk clotting efficiency, and reduced unwanted proteolysis in cheese by 60-80%. This enzyme engineering work resulted in a commercial product to be released by Chr. Hansen in 2019.

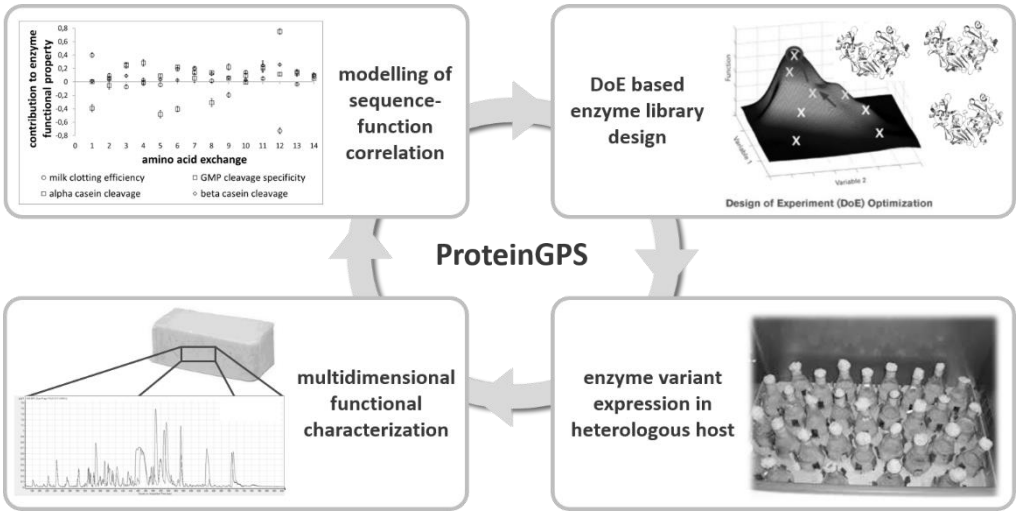

Figure 1: Multidimensional engineering of chymosin for cheese production by repetitive cycles of machine-learning guided directed evolution.

ARTIFICIAL METALLOENZYMES FOR IN VIVO CATALYSIS: CHALLENGES AND OPPORTUNITIES

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Key Words: artificial metalloenzymes, synthetic biology, artificial metabolism, directed evolution.

Artificial metalloenzymes (ArMs) result from the incorporation of a catalyst precursor within a host protein, Figure 1. The resulting hybrid catalysts display features that are reminiscent of both homogeneous catalysts and enzymes. The optimization of the catalytic performance of ArMs is achieved by combining both chemical- and genetic means. The versatility of this chemo-genetic optimization strategy will be illustrated with selected examples including: transfer-hydrogenation, C–H activation, olefin metathesis, hydroamination etc, Figure 1. With the aim of integrating artificial metalloenzymes *in vivo*, the second part of the talk will present our efforts to combine ArMs with natural enzymes to mimic essential features of the metabolism including: cascade reactions as well as up- and cross-regulation. Having identified the critical metabolites leading to ArM's inhibition, our efforts towards engineering enzyme cascades *in vivo* will be summarized.

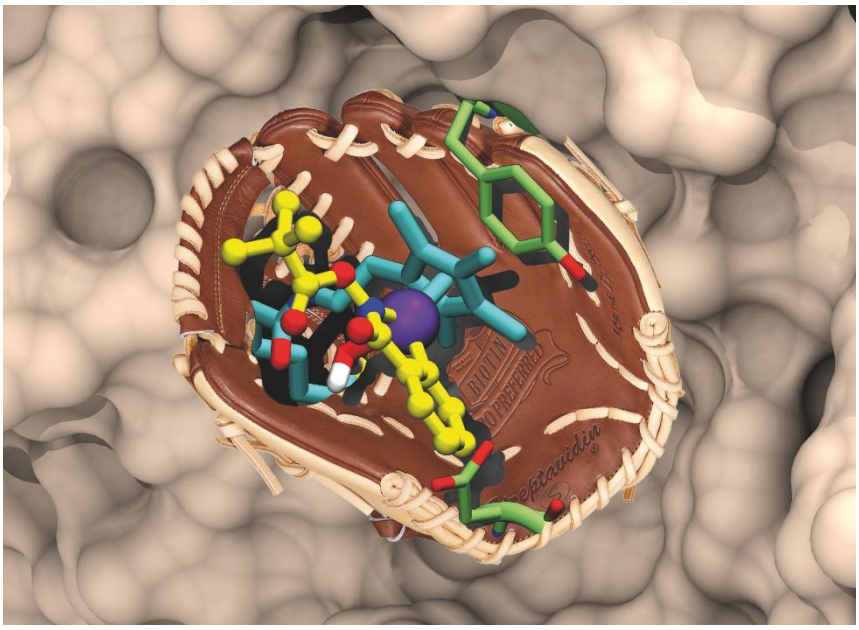

Figure 6 – Anchoring a catalyst precursor (ball & stick representation) within a host protein (baseball glove) affords an artificial metalloenzyme. The catalytic performance of the resulting hybrid catalyst can be optimized by chemo-genetic means: variation of the nature and position of the cofactor (turquoise stick representation) and mutation of aminoacid residues (green stick representation).

Reaction implemented
Hydrogenation
Transfer hydrogenation
Hydroamination
Allylic substitution
Suzuki cross-coupling
Dihydroxylation
Sulfoxidation
Alcohol oxidation
Peroxidation
Olefin metathesis
C–H activation
Cyclopropanation
Michael addition
Enzyme cascades

DESIGNING ARTIFICIAL METALLOENZYMES WITH HIGH ACTIVITY THROUGH ENGINEERING SECONDARY COORDINATION SPHERE INTERACTIONS

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Key Words: Artificial enzymes; metalloenzymes; biocatalysis; unnatural amino acids; non-native metallocofactors.

Metalloenzymes can catalyze some the most difficult and important reactions in biology. Designing artificial metalloenzymes (ArMs) with similar structure and activity as native enzymes is an ultimate test of our knowledge about metalloenzymes and can result in new biocatalysts for practical applications [1]. Despite progress made, most ArMs display much lower activity than native enzymes. A critical step to advancing the field is fundamental understanding what it takes to not only confer, but also fine-tune the ArM activity to as high as native enzymes. Only after we can demonstrate the ability to modulate ArM activity at-will to rival (or surpass!) natural enzymes can the potential of ArMs be fully realized.

A key to unlocking the ArM potential is the observation that the same primary coordination sphere (PCS) of a protein metal center can display diverse functions and a range of activity levels, leading to the realization that interactions in the secondary coordination sphere (SCS) are critically important. However, the SCS interactions, compared to the PCS structures, are numerous, long-range, and weak, making them very difficult to reproduce in ArMs, due to the sheer complexity of protein three-dimensional structure.

In this presentation, I will provide recent examples from Lu group and collaborators to demonstrate that, while reproducing the PCS may be good enough to make structural models of metalloenzymes, careful design of the non-covalent SCS interactions is required to create functional metalloenzymes with high activity. In the first example, we have demonstrated that, while a design of Cu_B center next to the heme in myoglobin that structurally mimics the heme-copper center in oxidases resulted in minimal oxidase activity, engineering water and associated hydrogen bonding networks next the PCS resulted in a dramatic increase in the activity [2]. When combined with further engineering of the electron transfer interface, the ArM has a comparable activity as native oxidase in solution [3] and 10 times faster activity as an electrocatalyst for the oxygen reduction reaction [4]. In the second example, we have shown that, while a design of [4Fe-4S] cluster next to the heme center in cytochrome c peroxidase that structurally mimics the heme-[4Fe-4S] center in sulfite reductase resulted in no activity, engineering SCS interactions to tune the reduction potentials of the [4Fe-4S] cluster and to enhance the substrate binding affinity resulted in an ArM with 20% of the native enzyme activity [5]. In the third example, through careful design of hydrophobicity and hydrogen bonding networks around the PCS of Type 1 copper center in azurin, we have been able to tune its reduction potentials to span across the entire 2V physiological reduction potential range [6], something that has not been achieved by any single class of native metalloproteins. Finally, we have shown that, by introducing unnatural amino acids [7], non-native metal ions [8] and metallocofactors [9], we have been able to fine-tune the activity of ArMs even better than those of native enzymes.

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IMMOBILIZING ORGANOMETALLIC CATALYSTS TO PROTEIN SCAFFOLDS FACILITATES TRACE-METAL REMOVAL POST-REACTION

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Key Words: Biohybrid catalysts, cascade reactions, biocatalysis, catalyst immobilization

Organometallic catalysts are used in a wide range of industrially important reactions. By immobilizing such catalysts to a protein (Fig. 1), we can bring them into aqueous media, fine-tune activities and selectivities and overcome the challenges associated with trace metal removal in the product fraction [1, 2]. Trace metal removal is particularly important for the synthesis of pharmaceutical compounds. Typically, the transition metal content should be below 10 ppm. We have employed two types of metal catalysts, a Ru-based Grubbs-Hoveyda-type catalyst for olefin metathesis and a Rh-catalyst for phenylacetylene polymerization. These catalysts were covalently attached to either nitrobindin (NB) [3] or *ferric hydroxamate uptake protein component: A* (FhuA) [4] β-barrel proteins, yielding biohybrid catalysts (also denoted artificial metalloproteins) that can be immersed in aqueous reaction media either in their free form or immobilized to bacterial cells. Moreover, we could show that the metal catalysts can be immobilized on surfaces consisting of silica or polypropylene via peptide-based adhesion promoters, thereby enabling “green” surface immobilization strategies with the potential of catalyst recycling [5]. Either strategy yielded highly active catalysts that show great promise for single or sequential one-pot reactions. Separation of products and the catalysts was readily achieved by extraction. With the potential to tune reaction efficiencies and selectivities by modifying either the metal catalyst or the protein surrounding, biohybrid catalysts bear great potential to amend or even substitute the repertoire of reactions available by organic synthesis and, likewise, biocatalysis.

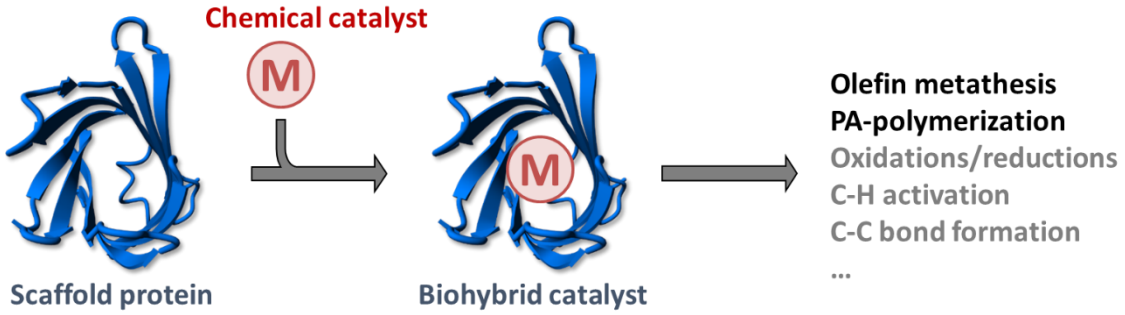

Figure 7 – Generation of Biohybrid catalysts by incorporating a metal catalyst into a protein scaffold.

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ENGINEERED MYOGLOBIN CATALYSTS FOR SELECTIVE CARBENE TRANSFER REACTIONS

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Key Words: hemoprotein; myoglobin; carbene transfer reactions; cyclopropanation; asymmetric synthesis.

Expanding the reaction scope of biological catalysts beyond the realm of enzymatic transformations occurring in nature can create new opportunities for the exploitation of biocatalysis for organic synthesis. In this lecture, we will present recent progress made by our group toward the design, investigation, and application of engineered myoglobins for catalyzing abiological carbene transfer reactions. These efforts have recently led to the development of efficient and stereoselective biocatalysts for the asymmetric construction of carbon-carbon and carbon-heteroatom bonds via carbene insertion into olefins, heteroatom-hydrogen bonds, C—H bonds, and carbonyls. These myoglobin-based catalysts could be successfully applied for the stereoselective synthesis of chiral building blocks and drug molecules at the multigram scale. Presentation of these results will be complemented with a discussion of our current understanding of the mechanism of these reactions and of the structural determinants of reactivity and stereoselectivity in this new class of ‘carbene transferases’.

ARTIFICIAL (BETA / ALPHA)$_8$ BARREL ENZYMES BY *IN VITRO* EVOLUTION

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Key Words: *de novo* enzyme, directed evolution, $(\beta\alpha)_8$ barrel fold, artificial protein

Natural evolution has yielded countless enzymes of the $(\beta/\alpha)_8$ barrel fold (TIM barrel). This versatile fold is highly favored by natural enzymes to catalyze a wide array of reactions and appears in five of the six enzymatic classes. Therefore, the $(\beta/\alpha)_8$ barrel fold is an attractive starting point for enzyme engineering. Several examples of modified TIM barrel enzymes have been reported that accept different substrates or even catalyze a different reaction. However, engineering new activities into the $(\beta/\alpha)_8$ barrel fold is apparently still far more challenging than its ubiquitous role in nature suggests.

We are interested in understanding the evolvability of enzymatic function in this fold. We established a generalizable *in vitro* selection approach for isolating new enzymatic activities without the need for prior mechanistic information. We created a protein library based on this fold by introducing seven randomized loops on the catalytic face of a monomeric, thermostable $(\beta/\alpha)_8$ barrel enzyme from *Thermotoga maritima* (GDPD). Because proper protein folding is a prerequisite for protein stability and activity, we pre-selected the library for protein folding by *in vitro* selection to increase the probability of finding new enzymes in the library. The resulting library contained ~10^{12} soluble monomeric $(\beta/\alpha)_8$ barrel protein variants [1]. We then used this library as the starting point to isolate novel enzymes through *in vitro* selection by mRNA display. We obtained artificial ligases enzymes that catalyze the joining of a 5'-triphosphorylated RNA to the 3'-hydroxyl of a second RNA. There are no natural enzymes known for this reaction; yet, we previously isolated enzymes for the same reaction from a non-catalytic zinc finger scaffold [2], and most recently also from a library of completely random polypeptides. We will present the detailed characterization of the new $(\beta/\alpha)_8$ barrel enzymes and contrast those to our other artificial ligases.

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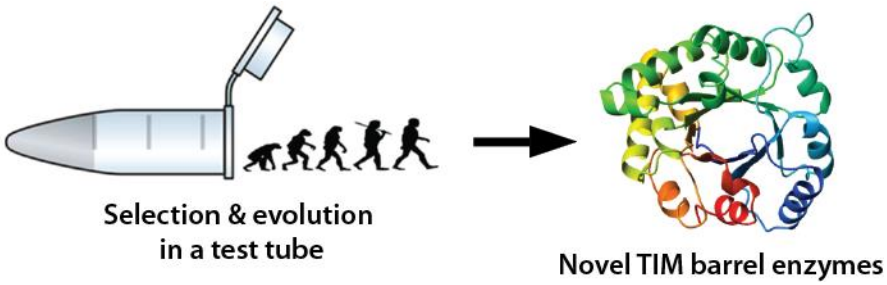

AN ARTIFICIAL METALLOENZYME FOR A BIMOLECULAR DIELS–ALDER REACTION

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Key Words: Diels–Alder metalloenzyme, computational design, directed evolution, enzyme catalysis, chemo- and stereoselectivity

The Diels–Alder reaction, one of the most important in organic chemistry, forms functionalized six-membered cycloadducts in a single step. While widely used to construct complex biologically active molecules in the laboratory, [4+2] cycloadditions are rarely employed for natural product biosynthesis in cells owing to the lack of appropriate enzymes. Creating artificial metalloenzymes able to exploit Lewis acid catalysis for substrate activation could change this situation. Embedding a metal ion in a chiral protein binding pocket potentially combines the best aspects of two worlds – transition metal and enzymatic catalysis – to achieve both high activity and selectivity. Here we report the transformation of a zinc-binding helical bundle into an artificial metalloenzyme that efficiently catalyzes a hetero-Diels–Alder reaction between 3-vinyl indole and an azachalcone derivative by a process of design and laboratory evolution. The best enzyme, DA7, performed >15,000 turnovers per active site and produced only a single product stereoisomer (>99% *ee*). Detailed kinetic analysis showed that this catalyst is more than two orders of magnitude more proficient than other known Diels–Alderases, including many designed catalysts and natural enzymes involved in polyketide natural products biosynthesis. The remarkable activity of DA7 can be ascribed to the Zn(II) ion, which activates the heterodiene for reaction, and a shape complementary binding pocket that preorganized the reactants for efficient reaction and exacting control over chemo-, diastereo-, and enantioselectivity. These results establish the feasibility of combining design and evolution to harness the structural and functional properties of metal ions to produce remarkably active enzymes for an important abiological reaction. Extending this approach to metal ions other than zinc, and to scaffolds beyond helical bundles, can be expected to produce proficient custom-metalloenzymes for a wide spectrum of unnatural chemical transformations.

GUIDING TRANSITION-METAL CATALYST SELECTIVITY USING PROTEINS

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Key Words: Artificial metalloenzymes, selective catalysis, sustainability

Artificial metalloenzymes aim to combine the benefits of natural enzymes (selectivity, rate enhancement) with the scope of reactions provided by traditional transition metal chemistry (both reactions inspired by nature e.g. oxidations and reactions never seen in nature i.e. Pd cross-coupling reactions).[1] Proteins containing apolar cavities and tunnels have been utilized as scaffolds in which transition metal complexes can be introduced to build up catalysts that provide linear selectivities and improve substrate turnover through enhancing substrate binding.[2, 3] A number of different methods can be utilized to introduce the metal complex into the protein scaffold, including either introducing reactive amino acids (e.g. cysteine's) which can subsequently be modified with a metal binding ligand, or directly introducing metal binding unnatural amino acids such as bipyridylalanine using amber stop codon suppression methodology.[4] In this talk, I will cover our work towards developing artificial metalloenzymes using these methods for a range of synthetic challenges from the biphasic linear selective hydroformylation of alkenes to selective C-H functionalization.

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CONSTRUCTION OF NOVEL METABOLIC PATHWAYS WITH ARTIFICIAL ENZYMES

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Key Words: Artificial Pathway, Artificial Enzyme, Rubber Materials, Alkaloids

Non-fossil raw materials can be utilized for the production of useful compounds by way of microbial "fermentation". Sugars are obtained from carbon fixations of plants or photosynthetic microorganisms, and are used as a carbon source for the biosynthesis of useful target compounds by genetically modified microorganisms. In order for a microorganism to produce enough target compound, techniques for optimal metabolic design must include balance of energy production/consumption, redox pathways, and intracellular carbon flow. With recent innovations in genome analysis technology and information processing technology, computational design tools that can describe more than 1000 genome-scale metabolic reactions to efficiently produce target compounds have been developed worldwide. However, the established tools are not designed to search and create biosynthetic pathways for production of non-natural compounds from fossil resources. We developed BioProV and M-path, new simulation tools that enable metabolic design for the biosynthesis of unnatural compounds. By combining these tools with enzyme engineering technology, we succeeded in expanding the scope of bioproduction targets. The first example is construction of an artificial metabolic pathway to biosynthesize isoprene. Isoprene the raw material for production of synthetic rubber that can be used in automobile tires. Currently, isoprene is industrially produced as a by-product of naphtha pyrolysis. Therefore, by establishing green isoprene production technology, dependence upon petroleum can be reduced. Isoprene is a substance that can exist within cells of many organisms as a monomer of polyisoprene rubber, and also as a structural unit of secondary metabolites. It is difficult to optimize its synthentic pathway due to shortages of intracellular ATP supply, and challenges in the introduction of improved biosynthetic pathways. In nature, isoprene is produced from mevalonic acid through a five-step reaction, but the newly constructed artificial metabolic pathway consists of just two steps from mevalonic acid to isoprene. This results in a three-fold reduction in cellular energy consumption. Furthermore, we succeeded in constructing a highly active enzyme that exhibits 10,000-fold higher isoprene-producing activity relative to natural enzymes. By introducing these artificial metabolic reactions into *Escherichia coli*, efficient artificial isoprene production was achieved. In addition, we have developed a microbial production system for 1,3-butadiene, another alternative source for synthetic rubber. Moreover, rationally engineered enzymes from insects and plants enzymes have resulted in the construction of an artificial pathway to benzylisoquinoline alkaloids and downstream opioid analgesics.

ENGINEERING DECARBOXYLASES FOR CONSOLIDATED BIOPROCESSING AND MORE

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Key Words: decarboxylase, thermostabilization, organic solvent, substrate promiscuity, biomass utilization

Decarboxylases have been widely applied for the production of chemical building blocks from biomass derivatives as well as chiral intermediates in organic synthesis.

First we have engineered different enzymes of this class (KDCA and PDC) for highly increased thermostability by combining rational design and random approaches. With melting points increased by almost 15 °C and half lifes above 70 °C prolonged up to several thousand fold this now opens up new possibilities for the production of alcohols such as ethanol, butanol or isobutanol from lignocellulose using thermophilic organisms in so called “consolidated bioprocessing” approaches, where lingo cellulose break-up is done simultaneously with product formation. In addition variants for higher tolerance towards denaturing, partially water miscible organic solvents were developed to be utilized e.g. for organic synthesis or in cell-free cascades like for the production of isobutanol from sugar.

Often, decarboxylases show broad substrate promiscuity, which can be of advantage but also a major challenge in fine chemical production. By further engineering the substrate specificity and modifying the promiscuity, it was possible to modulate the formation of desired chemicals with high selectivity. Based on this we have designed a network of artificial enzymatic cascade reactions towards production of bio-based building blocks utilizing decarboxylases as the modulating enzymes. With engineered variants, we demonstrate the conversion of industrial byproducts into several fine chemicals with high selectivity and yield. This result also sheds light on substrate recognition of the class of decarboxylases towards more-challenging substrates, e.g. bulkier side chains for broader applicability.

ENGINEERED ENZYMES, PATHWAYS, AND TOOLS FOR THE BIOSYNTHESIS OF NON-NATURAL POLYKETIDES AND TERPENES

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Key Words: polyketides, isoprenoids, terpenes, synthase, secondary metabolites

Many clinically used drugs are derived from secondary metabolites that are biosynthesized in a modular fashion by the selection and assembly of small molecule building blocks. Chimeric biosynthetic pathways can be constructed in an attempt to produce analogues for drug discovery. Yet, the scope and utility of this combinatorial approach is limited by the inherent substrate specificity and poor functional modularity of most biosynthetic machinery. Here, our approach to expanding the scope of polyketide and isoprenoid combinatorial biosynthesis by leveraging enzyme engineering and synthetic biology will be summarized. Our recent advances that realize the installation of multiple extender units into polyketides by engineered polyketide synthases will be presented, in addition to genetically encoded biosensors that enable directed evolution of natural product biosynthetic machinery in living cells. Furthermore, an artificial biosynthetic pathway for the biosynthesis of isoprenoids is described that utilizes non-natural building blocks and can support high titers of non-natural isoprenoids in *E. coli*. Our synthetic biology approach expands the synthetic capabilities of natural product diversification strategies and provides an improved understanding of the molecular basis for specificity in complex molecular assemblies.

NANOMACHINE BIOCATALYSTS: TOOLS FOR CELL-FREE ARTIFICIAL METABOLIC NETWORKS

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Key Words: nanomachines, biocatalysis, cell-free metabolic engineering, cofactor recycling.

Assembling cell-free, cascading multi-enzyme enzyme reactions into artificial metabolic networks for the conversion of low value renewable feedstocks into high value products represents a fourth wave of biocatalysis for renewable green chemistry and synthetic biology applications [1]. However, major limitations to both applications include the cost of producing multiple purified enzymes and of providing a continuous supply of diffusible cofactors or cosubstrates [2]. We have applied synthetic biology principles to produce fusion proteins between synthetic enzymes and their cofactor-recycling partner enzymes, with concomitant *in situ* recycling of a modified tethered cofactor, with an added conjugation protein element to allow immobilization of the nanomachines to a surface. This has enabled the construction of nanomachine flow reactors which can be combined in an interchangeable, “plug-and-play” manner to construct complex synthetic networks or Nanofactories. Synthesis of the anti-diabetic drug, D-fagomine, reductive amination to produce various chiral or conjugated amines (Fig. 1) and deracemization of alcohols have been used to exemplify the principles, and we have demonstrated tethered cofactor recycling of ATP, NAD(H)$^+$ and NADP(H)$^+$, as well as ligand-directed immobilization of a variety of enzymes to illustrate the use of these nanomachine biocatalysts as tools for the *de novo* construction of *in vitro* metabolic networks for synthetic biology. Our research is currently exploring the use of frugal innovation principles to integrate key capabilities in reactor design with on-line analytics for real-time reaction monitoring, and, subsequently, dynamic control over the platform’s fluidics via feedback loops. We aim to demonstrate the utility of such systems for cell-free metabolic engineering to enable fine chemical synthesis, with additional applications possible in bioremediation and environmental sensing.

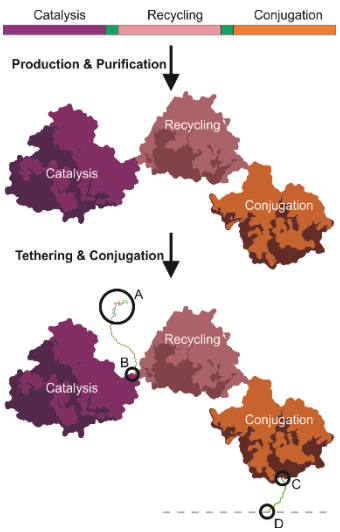

Figure 8 – Nanomachines: engineered enzymes that retain and regenerate their cofactors enable cell-free metabolic engineering.

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A DATA-DRIVEN APPROACH FOR EXPLOITING ENZYME PROMISCUITY AS A MEANS TO PREDICT NOVEL BIOCHEMICAL REACTIONS

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Key Words: machine learning, chemo-informatics, novel reactions, synthetic biology

Systems metabolic engineering has been widely used to produce chemicals of high commercial value from low cost substrates. But this process has challenges for some applications, such as harnessing lignocellulosic biomass for biofuel and biochemical production, due to our limited metabolic knowledgebase. With current advances in protein engineering, it is possible to exploit substrate promiscuity of enzymes to enable novel biochemical reactions. Nevertheless, performing experiments to determine what substrates an enzyme can act on can be time consuming and it is not always clear what potential substrates to test. So, the current work aims to employ machine learning approaches for identifying novel substrates and in turn, predicting novel reactions that are more promising than the putative reactions predicted simply based on compound similarity measures (e.g., Tanimoto coefficient). A highly accurate (up to 88.3%) machine learning model was developed to identify candidate substrates for *alcohol dehydrogenase* (ADH) using a dataset consisting of 23 metabolites (with 8 of them being known positives) and 46 chemo-informatics based molecular descriptors (e.g., topology, stereochemistry, and electronic features). In addition, support vector regression proved to be a useful method for estimating enzyme kinetics (characterized by Michaelis-Menten constants, K_m and V_{max}) for a variety of oxidoreductases that are typically found in biofuel biosynthesis pathways. Such machine learning methods can be applied to other classes of enzymes and hence, used as a tool to expand the knowledgebase of metabolic reactions paving the way for next generation of metabolic/ pathway engineering.

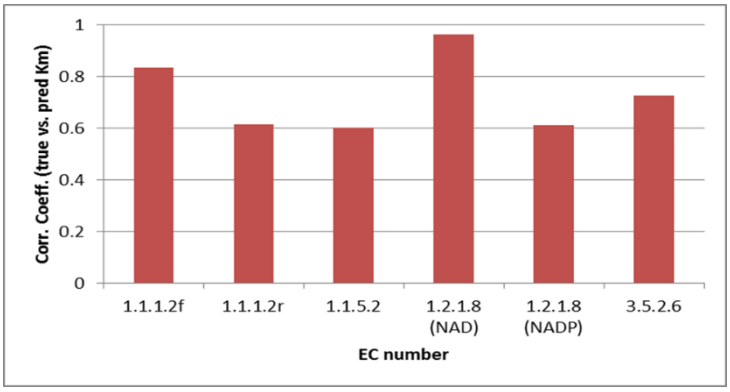

Figure 9 – Correlation coefficient b/w true and model predicted K_M values for different enzyme-substrate datasets [f - forward reaction; r - reverse reaction]

ENZYME ENGINEERING TOWARDS A FULLY BIOCATALYTIC MANUFACTURING ROUTE FOR MK-8591

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Key Words: HIV; MK-8591; manufacture; enzyme cascade; enzyme engineering.

With 37 million people around the world infected with HIV—and this number rising by almost 2 million a year—the search for newer, better medicines to treat this deadly disease remains an important goal. MK-8591 is a nucleoside reverse transcriptase inhibitor currently undergoing clinical trials for the treatment of HIV.

In order to achieve a world-class manufacturing route to this target that is also green and sustainable, the process chemistry team has turned to biocatalysis. Specifically, the 4'-ethynyl-2'-deoxyribose sugar portion of the molecule is prepared via a highly efficient three-enzyme cascade sequence. Another multi-enzyme reaction system is then used to append the 2-fluoroadenine base, overcoming a challenging glycosylation reaction.

This bold endeavor has posed numerous technical challenges in the optimization of complex multi-enzyme systems, the need for directed evolution of multiple enzymes, the detection and analysis of polar chromophore-free molecules and the isolation of unstable and highly water-soluble intermediates. An overview of the ongoing development and unique challenges—and opportunities—of this unprecedented fully-enzymatic manufacturing route will be presented.

ENGINEERING PET-DEGRADING ENZYMES FOR BIORECYCLING AND BIOREMEDIATION

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Key Words: plastic biodegradation, enzymatic recycling

Plastics, due to their inert properties and resistance to biodegradation, have been ravaging ecosystems worldwide and are especially harmful to aquatic wildlife. Plastics in the environment wear and tear into micron sized particles, termed microplastics, which are ingested and/or affect organisms at every level of the food chain. Recently, microplastics have been reported in human feces, and their potential health hazards remain unknown. In 2010, 4.8-12.7 million metric tons entered the oceans due to mismanagement and leakage, with an additional 31 million metric tons when terrestrial and freshwater ecosystems are considered. Both food and water supplies are likely contaminated with microplastics, and we need technologies to decrease formation of microplastics and remove these particulates from the environment. One of the most synthesized plastics is poly(ethylene terephthalate) (PET), an aromatic polyester with extremely low degradation rates. Due to the huge negative environmental impact of PET products, efficient recycling strategies need to be designed to “close the loop”’ to reduce dependence on petroleum feedstocks and decrease economic loss through single-use practices. The recent discovery of a PET-consuming bacteria *Ideonella sakaiensis* and its PET hydrolases has shown potential for enzyme-mediated recycling and bioremediation. Here, we present preliminary characterization of the catalytic rate of the newly discovered PETase and its behaviour over time, with perspective into future engineering potential for the enzyme for use in industrial processes.

NEWLY DISCOVERED ENZYMES AND CASCADES FOR THE DETERMINATION OF AMINO ACIDS

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Key Words: L-amino acid oxidase, L-amino acid dehydrogenase, micro-determination

The high stereo- and substrate specificities of enzymes have been utilized for micro-determination of amino acids. With the successful practical use of oxidoreductases such as the NAD^+-dependent L-Phe dehydrogenase for phenylketonuria among more than 5,400,000 of neonates in Japan [1], many enzymes with higher substrate specificities have been screened and discovered from Nature [2].

In this report, successful screening and uses of selective enzymes for micro-determination of amino acids will be discussed. Focuses on amino acid oxidases that are renewing the common sense of classical biochemistry will be described: L-Thr dehydrogenase [3], L-Trp dehydrogenase [4], L-Lys e-oxidase [5], L-Trp oxidase [6,7], L-Arg oxidase [8], Gly oxidase, Taurine oxygenase [9], etc, all of which show high selectivity.

In addition, novel enzymatic assays with the systematic use of cascades of known enzymes, including assays based on a pyrophosphate detection system using pyrophosphate dikinase for L-Met, L-Cit, L-Arg [10] and a variety of L-amino acids with amino-acyl-tRNA synthetase (AARS) [1,11] will be discussed. The X-ray crystallographic structures of some of the enzymes and the protein engineering are often necessary to use them practically. Together with the use of microbial determination of amino acids with lactic acid bacterium transformed with marker enzymes [12], most of the amino acids can be determined by the enzymatic methods (Table 1). The success and the potential for amino acid determination in biological samples will be shown.

Table 1. Amino acid determination by various enzymes and enzyme system

	Previous study	Our study				Previous study	Our study		
		Oxido-reductase	AARS assay	Bio-assay			Oxido-reductase	AARS assay	Bio-assay
Ala	●		●		Met		●	●	
Cys			●		Asn				
Asp			●		Pro			●	●
Glu	●			●	Gln				
Phe		●	●		Arg		●		●
Gly	●		●		Ser			●	
His	●		●	●	Thr		●		
Ile			●	●	Val			●	●
Lys	●	●	●		Trp		●	●	
Leu	●			●	Tyr		●		

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PROTEIN DYNAMICS AT SLOW TIMESCALES IN ENGINEERED ß-LACTAMASES DOES NOT LIMIT EVOLVABILITY

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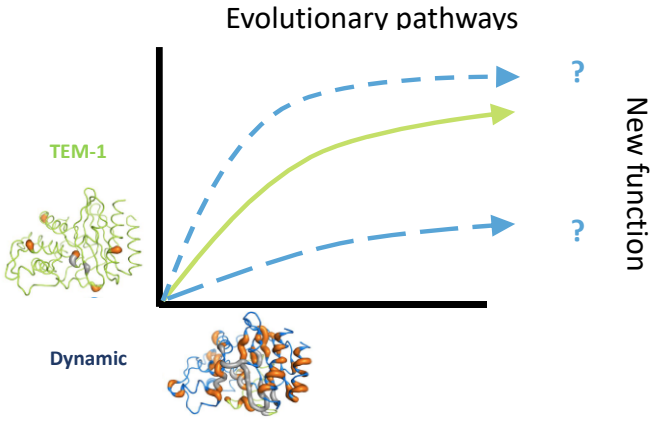
Key Words: Epistasis, protein dynamics, ß-lactamase

Understanding the underlying mechanisms in the evolution of new protein functions is key to better directing enzyme engineering efforts. Intragenic epistasis (the non-additive interaction of mutations affecting function) is a key feature of protein evolution. For example, in TEM-1 ß-lactamase, the mutations E104K and G238S show positive epistasis in that their combination show a greater than expected increase in antibiotic resistance[1]. Here, we aim to understand the impact of protein dynamics at slow timescales on epistasis. Large conformational rearrangements associated with ligand-binding, turnover of substrate or allostery occur at this timescale[2]. The readily evolvable antibiotic-resistant TEM-1 ß-lactamase and two of its variants that are more dynamic at slow timescales serve as models for this study. Our models show similar catalytic activity and substrate recognition, thermal stability, as well as conserved motions in fast (ps-ns) and intermediate (ns-μs) timescales but different motions at slow timescales (μs-ms)[123].

In this study, we use two different approaches to examine the effect of protein dynamics on epistasis. First, we introduced the epistatic mutations E104K and G238S into our dynamic variants. These mutations confer high resistance to the antibiotic cefotaxime in TEM-1, increasing catalytic efficiency ~250-fold. The dynamic variants present similar kinetic values and increase in catalytic efficiency as does TEM-1 when the epistatic mutations are introduced. Molecular dynamic simulations in the presence of cefotaxime support these observations, as the presence of the epistatic mutations correlates with an increase in catalytically-competent conformers. Secondly, we performed directed molecular evolution in TEM-1 and its dynamic variants towards the hydrolysis of the antibiotic cefotaxime. We examined whether mutational pathways accessible to TEM-1 are also available in the context of increased dynamics at the timescale of turnover.
Overall, our work highlights that protein dynamics at slow timescales does not hinder the evolution of new activity in TEM-1 ß-lactamase engineered variants. Furthermore, epistasis can be maintained despite differences in dynamics at slow timescales.



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ENZYME EVOLUTION IN SYNTHETIC BIOLOGY:
A KEY ROLE FROM PROOF-OF-CONCEPT TOWARDS PRECISION FUNCTION

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Enzyme evolution plays an important role in generating the novel catalytic functions of the enzymes for the desired reactions and thus provides a promising route for bio-manufactures. Here, we give examples for discovering and optimizing the promiscuous enzymes and exploring their uses in reprogramed biosynthetic pathways of Ginsenoside Rh2 and 2-Amino-1,3-propanediol. Through engineering the aminotransferases and the glycosyltransferase in vitro based on their catalytic mechanisms, we successfully repurposed the biosynthetic pathways for high production outcomes in vivo in hosts including Saccharomyces cerevisiae and Escherichia coli. These works demonstrate that enzyme evolution combined with the metabolic engineering is an efficient approach for environmentally friendly productions of pharmaceuticals and chemicals.

ENZYME DESIGN BY DIRECTED EVOLUTION

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