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Protein engineering for development of new hydrolytic biocatalysts

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Hydrolytic enzymes play important roles as biocatalysts in chemical synthesis. The chemical versatility and structurally sturdy features of *Candida antarctica* lipase B has placed this enzyme as a common utensil in the synthetic tool-box. In addition to catalyzing acyl transfer reactions, a number of promiscuous activities have been described recently. Some of these new enzyme activities have been amplified by mutagenesis. Epoxide hydrolases are of interest due to their potential as catalysts in asymmetric synthesis. This current update discusses recent development in the engineering of lipases and epoxide hydrolases aiming to generate new biocatalysts with refined features as compared to the wild-type enzymes. Reported progress in improvements in reaction atom economy from dynamic kinetic resolution or enantioconvergence are also included.

Introduction

The use of enzymes in organic synthesis is referred to as biocatalysis [see ref. 1 for a recent topic update]. There are clear advantages of applying enzymes in synthetic protocols. Enzymes are often unchallenged as catalysts thereby improving synthesis efficiency, economy, and provide a path towards more sustainable manufacturing of chemicals. One can tick off several of the points listed in the Principles for Green Chemistry [2] if enzymes were to replace traditional organochemical and metalloorganic catalysts at a wider scale.

The sources of enzymes that are utilized as biocatalyst are diverse and range from isolates from classical model organisms such as *Escherichia coli* or bakers yeast to extremophilic microbes and

metagenomes of particular microbiological societies [3, **4]. Additionally, contemporary methodologies for protein engineering have facilitated introduction of desired modifications to existing enzymes [**5]. This current update describes the recent progress of how different levels of protein (re-)engineering together with other optimizations, such as dynamic kinetic resolutions, in two important classes of hydrolytic enzymes, lipases and epoxide hydrolases, have contributed to the development of new useful biocatalysts (Figure 1) .

The biocatalysis community has been successful in isolating enzymes facilitating production of an increasing range of desired products but the majority of applied reaction types have been limited, with hydrolysis and acyl transfer reactions being the most widely adopted. Enzyme (lipase) catalyzed transacetylations are common practice today also within the organic chemistry

community. However, the underlying structure-activity related mechanisms that cause the desired product formation have been, if not neglected, of lower priority. Enzymologists, on the other hand, have produced structure-activity data on a range of important model enzymes that are often of low applied value as biocatalysts. This has led to a knowledge gap between these disciplines, one more applied and the other fundamental. An attempt to address this issue and contribute to bridging the gap was the STRENDa initiative [7] which described guidelines for presentation of functional data in isolated biocatalysts.

Lipases

Lipases A and B from *Candida antarctica* have historically been the most applied biocatalysts and for many non-biochemists the quintessential representatives of enzymes in general. The success of lipase B (CALB) as biocatalyst can be considered as something of a mixed blessing for the field. This enzyme displays, on one hand, many desirable properties, both functionally and physicochemically. It is remarkably stable in various organic solvents which renders it perfect as catalyst of transesterification reactions [8] and also allows for solubilization of hydrophobic reactants and products. CALB is also relatively thermostable which further affects reaction rates favorably. These properties, especially the stability in non-polar solvents are, on the other hand, quite unique and enzymes in general do not accept such an environment. (Hence, CALB is not a good representative of enzymes in general and the realization of the synthetic chemist that most enzymes denature and lose catalytic activity under such conditions can be off-putting when designing a synthetic strategy and biocatalytic approaches may be excluded.)

CALB has been primarily applied in catalysis of transesterification reactions but also displays promiscuous activities. Various protein engineering efforts in recent years have targeted improved catalytic efficiencies and altered substrate and stereoselectivities. A noteworthy contribution was engineering by circular permutation [9] where one enzyme variant (dubbed cp283) exhibited improved hydrolysis activity with esters **1a-1c** (Table 1) [10]. The reason for the activity increase

was traced to a structural rearrangement of a loop that allowed for faster substrate entrance and product exit in the mutant [*11]. In a CALB-catalyzed promiscuous reaction, an S105A point mutant (S105 is the catalytic nucleophile otherwise required for acyl transfer reactions) catalyzed C-C bond formation in Michael addition reactions (Scheme 1) [12] as well as hydrogen peroxide afforded epoxidation of α,β -unsaturated substrates [13]. Further, in a combined bioinformatics and computational study mapping amino acid residues required for hydrolytic activity in structurally related amidases and lipases of the α/β -hydrolase superfamily, candidate residues responsible for amidase activity were

identified. Although catalytic groups were superimposable between the compared amidases and lipases, structural differences in neighboring loops were observed. When corresponding mutations were introduced into CALB, enzyme variants displaying up to 11-fold improvement in hydrolytic activity with **2** were identified [14]. In another study, also targeting to improve upon the miniscule amidase activity of wild-type CALB, a substrate-contributed hydrogen bond proposed to lower the activation barrier of cleavage of the scissile C-N bond was used as role model for protein engineering [15]. Residue I189 was mutated into residues potentially capable of contributing hydrogen bonds, and might thereby facilitate amidase activity [16]. Both the I189Q and I189N variants did indeed exhibit increased preferences for hydrolysis of **3** as compared to the corresponding ester analog.

Kinetic resolution of accepted substrates is an often applied approach to yield production of enriched enantiomeric excess of products. A drawback with this strategy is the maximum 50% conversion of starting material into desired product. A more desirable strategy would result in full

conversion but require enantioconvergence or dynamic kinetic resolution. The latter has been reported for a CALB-mutant (W104A) that catalyzed transacetylation of phenyl substituted *sec*-alcohols. The mutation allows for larger substrates than ethyl-substituted derivatives to be accepted and also changes the the enzyme's stereoselectivity to prefer (*S*)-enantiomers. A rhutenium-based metalloorganic catalyst was included to catalyze racemization of remaining alcohol thereby refilling the depleted (*S*)-enantiomer of the reactant [*17]. The same strategy has been described for chemoenzymatic synthesis of cyclic ketones [18].

CALB displays poor activity with chiral α -substituted esters, some of which are precursors for synthesis of *e.g.* ibuprofen derivatives. Reetz and co-workers conducted ISM-driven directed evolution selecting for enzyme variants with activity and stereoselectivity in hydrolysis either enantiomer of **4** [**19]. A number of active variants were isolated which displayed activities also with other, non-selected for, α -substituted esters.

CALB has also been utilized as catalyst for synthesis of lactate-based polymers, polylactides. A triple mutant (Q157A, I189A, L278A) was designed after modeling and MD simulations of the putative acylenzyme intermediate. The replacements were aimed to minimize sterical constrains in the active site thereby facilitating the cyclic propagation of oligolactide synthesis. Although the mutant displayed lowered activity with a standard CALB substrate, ethyl octanoate, it was substantially more efficient in catalyzing both initiation and propagation of polymer synthesis, as compared to the wild-type enzyme [*20]. In another modeling-guided engineering of CALB, variants were constructed that showed increased activity in diester formation between ethane-1,2-diol or butane-1,4-diol and acrylic esters [21].

Lipase A (CALA) from the same yeast has been less extensively studied or applied as biocatalyst, as compared to CALB, but recent work has aimed to modulate this enzyme to improve on its biocatalytic usage. Semi-rational directed evolution of the enzyme active site generated variants with improved enantioselectivity towards either enantiomer of **5**. The observed

improvements were caused by different mechanisms. In one case, decreased activity with the unfavored enantiomer (*R*) together with retained activity with (*S*)-**5** resulted in increased overall (*S*)-selectivity. In another instance, a mutant that had acquired (*R*)-preference was achieved that as a results of the combination of decreased activity with (*S*)-**5** accompanied by an increased activity (*R*)-**5** [22]. The same research group went on to improve on the enantioselectivity of CALA in hydrolysis of α -substituted carboxylic acid esters. One isolated triple mutant (F149Y, I150N and F233G) catalyzed hydrolysis of derivatives of **6** reaching impressive E-values (>200) and enantiomeric product excesses of >98% (*R*)-enantiomer from kinetic resolution of the racemic ester [23]. Further structure-model-guided mutagenesis and directed evolution of CALA, applied a radically decreased codon subset during mutagenesis and thereby allowed for visits to a larger number of active-site residues. The approach was successful in producing variants capable of hydrolysis of ibuprofen ester **7**, generating (*S*)-ibuprofen in high enantiomeric excess [24].

A QM/MM study by Fruschicheva and Warshel applied the empirical valence bond method to assess the feasibility to mimic the enantioselectivity of wild-type CALA and a selected set of mutants [25]. The results were encouraging in that the trends of either (*R*) or (*S*)-preference could be modeled. The authors stress, however, the importance of extensive sampling in order to reach acceptable accuracy.

In a study on related lipases from *Candida rugosa*, the authors could pin-point a single residue in two isoenzymes that was decisive for enantioselectivity in the hydrolysis of **8** [26]. Replacing a small amino acid residue (Ala or Gly) for a bulkier (Val, Leu or Phe) shifted the enantiopreference from the (*R*)-enantiomer to the (*S*)-enantiomer.

Epoxide hydrolases

This class of hydrolases have been a subject of much interest due to their potential as biocatalysts in asymmetric synthesis of vicinal diols [27-30]. Recent progress in understanding the mechanisms that decide reaction outcome and catalytic efficiencies are now focusing more on

guiding protein engineering aiming to improve biocatalyst properties and performance.

Reetz and co-workers improved the enantioselectivity in the hydrolysis of **9** from 4 to 115 by five steps of ISM-driven directed evolution [31]. The improvement resulted in efficient kinetic resolution of the (*S*)-diol product and was primarily caused by a drastically decreased activity with (*R*)-**9**. The reason for the lowered activity with this enantiomer was explained by steric clashes from side-chains of introduced mutated active-site residues. My group have studied the potato epoxide hydrolase StEH1 regarding the enzyme's stereoselectivities, including enantio- as well as regioselectivity. Our present view is that these are truly plastic features [32-34] and can be engineered by active-site mutagenesis [35, 36]. A variant that had acquired five mutations in non-catalytic active-site residues through three rounds of directed evolution displayed a shift in regioselectivity in epoxide ring opening of (*S*)-**10**, while retaining the selectivity with the (*R*)-enantiomer, hence resulting in enantioconvergence of the diol product (80% *ee*_p). A similar study on an epoxide hydrolase from *Aspergillus niger* M200 resulted in a mutant that after having accumulated nine residue replacements afforded the formation of the (*S*)-enantiomers of the hydrolysis products of styrene oxide to an enantiomeric excess of 70% [37]. The ability of epoxide hydrolases to produce enantiomerically pure products from racemic starting epoxides by enantioconvergence was reported already ten years ago by the Furstoss group who analyzed the product outcome from StEH1 catalyzed hydrolysis of styrene oxide derivatives [38]. In a recent report, a similar behavior has been observed in another plant-derived isoenzyme from *Vigna radiata* that produces the (*R*)-diol product with an enantiomeric product excess of 70% from a racemic mixture of 4-nitrostyrene oxide [39].

Most work have been performed on epoxide hydrolase isoenzymes from the α/β -hydrolase and limonene epoxide hydrolase-fold families [30]. Recently, however, new enzymes from *Rhodococcus opacus*, belonging to the haloacid dehydrogenase structural superfamily, have been isolated and characterized [40-42]. The chemical mechanism is believed to proceed via a covalent

enzyme intermediate formed after nucleophilic attack of an enzyme carboxylate (D18), followed by general-base assisted (H190) hydrolysis. The proposed mechanism was concluded from results of ^{18}O labeling of the product which required multiple enzyme turnovers to incorporate the heavier isotope [40]. The mechanism is analogous to that established for the α/β -hydrolase isoenzymes.

Conclusions

Applying enzymes as chemical catalysts in synthetic protocols have proven successful. The possibilities to implement and refine a desired functional property by mutagenesis further increases the value of enzymes as synthetic tools.

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Table 1. Compounds mentioned in the text

Number referred to in text	Formula	Ref.
1a		10
1b		10
1c		10
2		14
3		16
4		19
5		25
6		23
7		24
8		26
9		31
10		35, 36