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Engineering lipase B from *Candida antarctica*

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Abstract—The lipase B from *Candida antarctica* (CAL-B) displays high enantioselectivity on a broad range of substrates, making it an accepted biocatalyst for asymmetric organic chemistry. In recent years, a number of rational and combinatorial protein engineering projects have focused on extending and tailoring CAL-B's catalytic and physical properties. Beyond generating customized catalysts, these studies have helped to elucidate the enzyme's structure–function relationship and illuminate its enantioselectivity. Potential directions for future studies, taking into consideration results from engineering efforts on related lipases, are discussed.

Contents

1. Introduction

Lipases (EC 3.1.1.3) play an important role in asymmetric biocatalysis. These enzymes possess broad substrate specificity, generally high regio- and enantioselectivity, and in many cases perform under mild conditions.[1] Applications include the kinetic resolution of racemic esters and amides, as well as the desymmetrization of prochiral compounds. In addition, they are successfully employed in selective esterification, transesterification, and polymerization reactions.[1–4]

Depending on the chemistry and desired reaction conditions, a substrate for asymmetric biocatalysis typically requires the screening of multiple lipases to identify the most suitable enzyme. Traditionally, this tailoring process has relied on collections of lipases, isolated from various natural or recombinant sources. More recently, these collections have been extended to include sources such as metagenomic DNA.[5,6] Further optimization of the reactivity and selectivity of promising catalysts is achieved by changes in the medium (immobilization, solvent, water content) and reaction conditions (e.g., temperature, pH, pressure).[7,8]

With the development of robust techniques for protein engineering, a third approach to match enzymes and substrates has emerged. Rational and combinatorial methods such as site-directed and random mutagenesis, as well as DNA shuffling in combination with powerful selection and high-throughput screens have

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revolutionized the search for customized biocatalysts.[9,10] When applied to lipases, these approaches offer the opportunity to adjust substrate- and enantioselectivity, as well as to optimize physical properties, such as thermostability and cofactor dependence.[2,11]

Among the lipase family members, lipase B from *Candida antarctica* (CAL-B) has shown outstanding biocatalytic characteristics for the stereoselective conversion of secondary alcohols.[8,12] The enzyme's exquisite enantioselectivity and tolerance for a broad range of substrates ensure that it has found extensive use in asymmetric biocatalysis. Yet despite its importance to organic synthesis and biotechnology, CAL-B only recently joined the small group of selected lipases targeted by protein engineering.

2. CAL-B: structural framework and catalysis

Knowledge of a protein's structure is a critical element in the structure–function analysis of engineered variants. The crystal structure of the wild-type CAL-B has been reported by Uppenberg et al.[13,14] Despite its low protein sequence homology to other known lipases and esterases, CAL-B assumes the characteristic α/β-hydrolase fold and presents the typical Ser-His-Asp catalytic triad in the active site (Fig. 1).[1] Co-crystallized substrate analogues further reveal the position of the transition state-stabilizing oxyanion hole (Gly39, Thr40) and help define residues critical for substrate binding.[14,15] The high stereoselectivity of CAL-B, in particular for secondary alcohols, is explained by a constricted binding pocket made up of Thr42, Ser47, and Trp104 (Fig. 1). Experimental data have shown CAL-B's preference for small and medium-size substituents (methyl = ethyl > isopropyl ≫ methylmethoxy) in the corresponding position (M_L) of the secondary alcohol. Contrasting the limited tolerance for the small side chain (M_L), CAL-B has relaxed specificity toward the larger alcohol fragment (L_L), and the carboxylate portion of the substrate (R) (reviewed by Ref. 1). In addition to the size limitations in the alcohol binding pocket, enantio-preference is further controlled through the optimal geometric orientation of Nε(His224) and the oxygen of the alcoholic leaving group, minimizing the energy barrier for the required hydrogen transfer (Fig. 1).

3. Engineering CAL-B

3.1. Rational protein engineering

Over the last decade, CAL-B's crystal structures in combination with phylogenetic analysis and computational methods have guided several rational engineering studies. Generally, these experiments have concentrated on amino acids in proximity to the active site. Critics may argue that such targeted studies reflect our limited ability to assess in silico the influence of distant residues on the active site. In contrast, Kazlauskas and co-workers recently suggested that local manipulations may indeed prove more effective toward changing enzyme properties.[16]

An early example of protein engineering of CAL-B by site-directed mutagenesis was the substitution of T103 for glycine.[17] The exchange experiment was inspired by data from sequence alignments of multiple lipases, revealing a glycine-rich consensus sequence surrounding the active site S105 (yellow region in Fig. 2). The T103G switch resulted in mutant protein with a substantially increased thermostability and only a twofold reduction in activity. A follow-up study revealed that the substitution caused no changes in substrate specificity or enantioselectivity.[18] The stability increase was explained by a reduction of local steric clashes and the formation of additional stabilizing contacts among structural elements in the protein.[18,19]

Extending upon the idea of adjusting CAL-B to the lipase consensus sequence, Patkar et al. also investigated the impact of substituting the adjacent W104 to histidine.[18] W104 is critical to the formation of the small substrate-binding pocket and its replacement with a smaller substituent caused a marked reduction in the enzyme's activity and enantioselectivity. These changes were largely attributed to a consequent enlargement of the binding pocket and possible global structural perturbation of the enzyme. The results were confirmed in a more recent study by Rotticci et al.[20] Under the premise of improving CAL-B's enantioselectivity toward halohydrins, the authors mutated the three key residues in the substrate-binding pocket: T42, S47, and W104. The positions were chosen based on computational studies that suggested that a combination of unfavor-

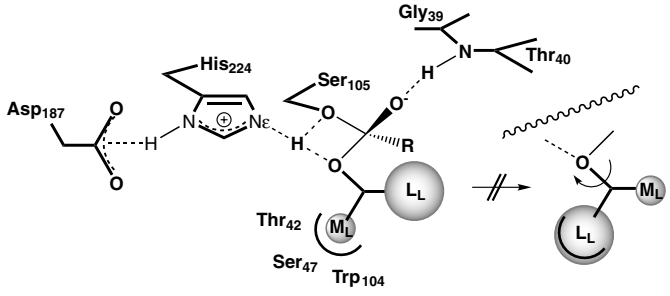


Figure 1. CAL-B's active site geometry and size restrictions for the binding orientation of the secondary alcohol largely control its enantioselectivity.

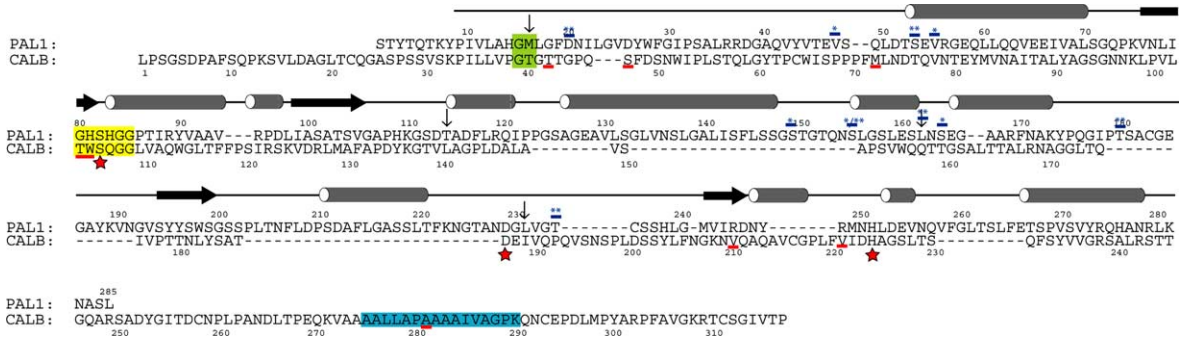


Figure 2. Summary of functional regions and mutation sites in *C. antarctica* lipase B (CAL-B) and *P. aeruginosa* lipase (PAL1). The sequence alignment is based on the structural overlay (CAL-B: 1TCA;[14] PAL1: 1EX9)[38] by CE.[39] Secondary structure elements are represented by tubes (helix) and arrows (sheet). The red stars identify the positions of the catalytic triad. The glycine-rich consensus sequence is indicated in yellow. Highlighted in green is the GX-motif, which is involved in oxyanion stabilization. The blue region specifies the unusually alanine-rich α-helix in CAL-B. The arrows (↓) mark the amino acid positions, which correspond to the site of limited mutagenesis in *Bc* KWI-56.[35] Underlined residues indicate positions of mutations discussed in the text (red—CAL-B; blue—PAL1).

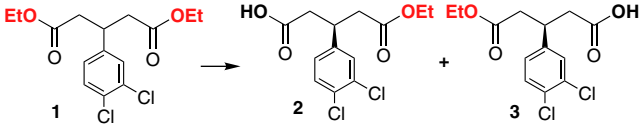
able electrostatic interactions and steric limitations was responsible for the observed moderate to poor stereoselectivity of the wild-type enzyme for this particular substrate. Individual, as well as combined substitutions of T42 and S47 to valine and alanine, respectively, raised the catalyst's enantioselectivity toward halohydrins twofold but lowered the specific activities by two- to fivefold.[20]

3.2. Combinatorial approaches to CAL-B engineering

Combinatorial methods for protein engineering are increasingly applied for tailoring biocatalysts. In the case of CAL-B, two recent manuscripts have reported improved thermostability and catalytic activity by random mutagenesis and DNA shuffling.[21,22]

In the first report, Zhang et al. employed error-prone PCR (epPCR), as well as site-directed and site-specific saturation mutagenesis to generate libraries of CAL-B, which were tested for variants with improved thermal properties.[21] Three consecutive rounds of mutagenesis and screening led to the identification of two mutants, 23G5 (V210I/A281E) and 195F1 (V210I/A281E/V221D), with up to 30-fold increased thermo-resistance. By selecting for enzyme activity *after* incubation at elevated temperature, the author's experiments identified mutants, which actually had lower temperatures of unfolding (T_{un}) than the wild-type enzyme but whose unfolding was reversible (in contrast to the parental enzyme). Rather than contributing to the overall stabilization of the protein framework, as observed for the T103G mutant,[17] the changes in 23G5 and 195F1 seemed to facilitate effective refolding of the mutant enzyme after heat denaturation. The significance of the individual amino acid substitutions was investigated.[21] Sequence analysis of CAL-B led to the hypothesis that A281 is part of an extended hydrophobic surface region (blue region in Fig. 2), which serves as a nucleation site for aggregation. The A→E substitution was proposed to disrupt the pattern and thereby facilitate refolding. A similar but weaker effect was predicted for V221 while V210's contribution was believed to be irrelevant. Experimental studies on site-directed mutants of all three positions confirmed that A281E is the primary factor responsible for the observed folding changes.[17]

More recently, a family DNA shuffling experiment involving the lipase genes from *C. antarctica*, *Hyphozyma* sp. CBS 648.91, and the thermophilic organism *Crytococcus tsukubaensis* was described.[22] All three parents display low activity for the prochiral diethyl-3-(3′,4′-dichlorophenyl)glutarate **1** (DDG) but greater than 99% ee for the (*S*)-isomer **2**. Library screening identified 69 clones (~2.7% of the tested library) with increased hydrolytic activity for **1** (Scheme 1). The subsequent in vitro analysis of seven selected hybrids confirmed a maximum 22-fold improved catalytic performance with retention of high enantioselectivity. Furthermore, the thermostability of the analyzed progeny was raised slightly, reflecting the range of parental T_{un}s.



Scheme 1. Lipase-catalyzed enantioselective hydrolysis of prochiral DDG **1** into the (*S*)-**2** or (*R*)-isomer **3** of the monoglutarate.

Sequence analysis of these functional hybrids led to speculations as to the basis of the observed improvements in catalysis and thermostability. For example, hybrid 3A4, one of the best DDG catalysts in this study, consists of the *Hyphozyma* sequence with an α-helical fragment from *C. tsukubaensis*, spanning the region, which corresponds to the hydrophobic alanine-rich sequence in *C. antarctica* (Fig. 2). Besides tendencies toward aggregation, several residues in this helical region are proximal to the active site, forming parts of the substrate-binding pocket. Amino acid mutations are likely to impact both functions. However, in the absence of detailed information on the binding properties of the parental enzymes and follow-up studies to investigate the effects of individual substitutions, conclusions such as those above remain highly speculative at best. Moreover, general trends among the selected library members

may need further evaluation. The analyzed samples indicate that fusion proteins with crossovers in the C-terminal half of the sequence seem favored among selected hybrids. Also, the library seems to be dominated by the lipase gene from *C. tsukubaensis* while only a few fragments from CAL-B were identified. Whether these biases reflect a selective advantage of the phenotype or are simply sequence-dependent phenomena as a result of shuffling, remains to be determined. Finally, the authors mentioned two selected candidates with inverted enantioselectivity. Despite the modest reported selectivity (55–60% ee) for the (*R*)-isomer **3**, these findings are highly significant in the context of function and sequence composition. Unfortunately, no further details have been reported on these library members.

In summary, the current engineering efforts on CAL-B, though limited in scope, clearly indicate the enzyme's potential for further functional improvements. This prediction is further supported by an extensive collection of results concerning the engineering of the α/β-hydrolase framework in general and lipase in particular.

4. Unexplored methods and potential directions for functional improved CAL-B

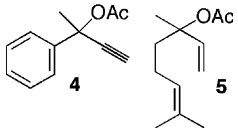
Engineering of CAL-B is not limited just to epPCR and DNA shuffling. Due to its low sequence identity compared to other lipases, which restricts DNA shuffling, a promising alternative for manipulating CAL-B's substrate and enantioselectivity would be the creation of hybrid proteins through domain swapping.[23–25] New opportunities also arise from advanced DNA shuffling techniques. Reetz and co-workers recently reported an oligonucleotide-based gene recombination method for the diversification of lipases from *Bacillus subtilis*, which led to the identification of variants with increased enantioselectivity.[26] Library construction could also be assisted by more efficient transformation techniques such as in vivo homologous recombination.[27]

While new methods are important for constructing new and more diverse libraries, the research focus should remain on how to improve the functional properties of proteins in general and lipases such as CAL-B in particular. Three selected examples for such functional challenges will be discussed in more detail: (a) the expansion of lipase substrate specificity to tertiary alcohols; (b) the improvement of enantioselectivity, and (c) the variation of enantio-preference within the α/β-hydrolase framework. All three questions have been studied in various members of the lipase-family and the solutions demonstrate the opportunities for successfully tailoring these biocatalysts.

4.1. Expanding substrate specificity

An interesting challenge for lipase engineering is the expansion of substrate specificity for utilizing tertiary alcohols. Found in natural products and industrial intermediates, chiral tertiary alcohols are generally poor substrates for lipases.[28] Specifically, CAL-B shows no detectable activity on a range of tertiary alcohols.[1,12,29] The small subfamily of enzymes that is actually capable of turning over the bulkier substrates shares a glycine-rich motif (GGGX), which spans the loop region of oxyanion-stabilizing residues.[28,30] The underlined amino acid indicates the position of the oxyanion hole. In contrast, the majority of lipases and esterases, including CAL-B, display a shorter GX motif in the same position (green region in Fig. 2). Mechanistically, the GGGX-motif is believed to translate into greater loop flexibility in comparison to the GX-sequence. The increased flexibility was speculated to minimize potential steric interference between the substrate and the catalyst's backbone. At the same time, this structural relaxation may explain the generally low enantioselectivity of GGGX-hydrolases (*E*-values ~1–10).[31]

Current engineering efforts have focused on the improvement of the enantioselectivity of GGGX family members. Henke et al. described a successful computer-aided protein engineering study on *p*-nitrobenzyl esterase from *B. subtilis*.[31] The combined modeling and experimental studies identified the first glycine in the GGGX-motif near the active site to be critical for the enzyme's enantioselectivity. In agreement with the computational predictions, a G→A mutation at that position resulted in a sixfold improvement in the enantioselectivity for 2-phenyl-3-butin-2-yl acetate **4** and an inversion of the enantio-preference (*R*→*S*) for linalyl acetate **5** (Scheme 2). Less desirably, the substitution reduced the mutant's catalytic activity 10-fold. Nevertheless, these results are encouraging examples of our increased understanding of factors that determine enantioselectivity in lipases. It remains unanswered as to whether similar changes in GX-family members could convert them to GGGX-like enzymes with expanded substrate specificity. Rational engineering of the GX motif in combination with combinatorial or computational methods to fine-tune neighboring residues could provide further insight into the structural changes required for extended substrate specificity.



Scheme 2. Tertiary alcohols as lipase substrates.

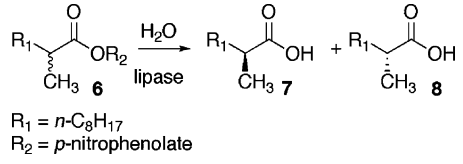
4.2. Improving and inverting enantioselectivity

The alteration of CAL-B's enantioselectivity by combinatorial methods represents another aspect of protein engineering that remains largely unexplored in literature. Such an endeavor may be considered unnecessary, given that the wild-type enzyme displays an impressive stereoselectivity over a wide range of substrates and that a large number of alternative lipases with potentially superior specificity are commercially available to fill the gaps.[1,12] However, advantageous structural features such as the small lid, which invalidates the need for interfacial activation, as well as the enzyme's high cata-

lytic activity do make CAL-B an attractive framework for remodeling.

The optimization of an enzyme's enantioselectivity can involve either the fine-tuning of an existing specificity to a higher *E*-value or, more dramatically, the inversion of the enantio-preference of the catalyst. As discussed earlier, rational changes in the substrate-binding pocket of CAL-B successfully raised the enantioselectivity of halohydrin hydrolysis twofold.[20] However, recent engineering studies on lipases from *Pseudomonas aeruginosa* (PAL1)[32–34] and *Burkholderia cepacia* KWI-56[35] have demonstrated that more extensive diversification involving the entire protein framework is capable of introducing more substantial changes to these enzyme's catalytic properties. These data, in combination with structure-based sequence alignments of PAL1 and CAL-B (Fig. 2), can facilitate the identification of potentially interesting amino acids in CAL-B.

In the case of PAL1, random and site-directed mutagenesis in combination with high-throughput screening led to the identification of lipase variants that could hydrolyze the *p*-nitrophenyl ester of 2-methyldecanoic acid **6** with an enantioselectivity of $E_S = 25.8$ (Scheme 3).[32,33] In comparison, wild-type PAL1 displays virtually no selectivity for this particular substrate ($E_S = 1.1$). Sequence analysis of the best-performing variant revealed five point mutations (V47G, V55G, S149G, S155F, and S164G; Fig. 2). Superimposing these residues on the PAL1 crystal structure revealed that none of the substitutions was proximal to the active site. Instead, the mutations were believed to modulate the enantioselectivity indirectly through conformational rearrangements. The importance of such structural changes was further emphasized by the accumulation of glycine residues in four out of the five mutated residues, resulting in an apparent increase in protein backbone flexibility. In the context of CAL-B, positions 149 and 155 in PAL1 are part of the missing lid structure in the yeast protein (Fig. 2). The three remaining amino acids are semi-conserved in the two proteins and may be important for the hydrolase-fold's dynamic properties.



Scheme 3. Hydrolysis of *p*-nitrophenyl-2-methyldecanoate **6** to the (*S*)-**7** or (*R*)-isomer **8** of the free carboxylate.

A second generation of laboratory evolution experiments led to a PAL1 variant with an even higher *E*-value for hydrolysis of **6**.[36] Combined cassette mutagenesis and DNA shuffling resulted in a lipase that carries six mutations (D20N, S53P, S155M, L162G, T180I, T234S; Fig. 2) and displays an E_S-value of 51. Homology modeling indicated that only position 162 was in close proximity (<10 Å) to the active site. Further insight in the effects of these mutations was gained from molecular dynamics simulations.[37] The results from these studies attributed the major contribution to enantioselectivity to residues 53, 155, and 162. The amino acids at these locations were suggested to undergo concerted rearrangements, forming steric relays, which, upon mutation, could indirectly affect binding of the substrate isomers in a catalytically competent conformation.

Rational and combinatorial methods have also been successfully employed to invert the enantio-preference of lipases and esterases. For PAL1, a variant with 11 mutations was identified in libraries generated by ep-PCR and DNA shuffling.[34] While the substitutions caused a switch to high selectivity for **8** ($E_R = 30$), the position of the mutations in the protein and the nature of the new side chains did not allow for a rational explanation. Their distances to the active site would certainly favor an explanation similar to that of the steric relay discussed above.

In contrast, a more targeted approach was chosen for the enantio-inversion of *B. cepacia* KWI-56 lipase.[35] The authors used restricted random mutagenesis to substitute four positions in proximity to the active site with a limited set of hydrophobic amino acids (Fig. 2). The library of ~2400 mutants was screened for variants with selectivity toward the (*R*)-isomer of *p*-nitrophenyl 3-phenylbutyrate. It identified 32 variants with E_R-values of up to 38, a complete reversal of the wild-type's selectivity ($E_S = 33$). While speculations on the impact of the mutations were discussed in the paper, detailed studies on the individual residues' contributions are required to understand their functional consequences. However, the paper demonstrates that enantio-inversion can be accomplished with a surprisingly small number of substitutions. Provocatively, the authors speculated that their semi-rational approach results in a conceptionally different yet qualitatively equal solution for enantio-inversion. While the evolutionary approach for PAL1 achieved enantio-inversion by altering the overall dynamic properties of the protein framework (mutations distant to the active site), the same characteristic was accomplished through conservative amino acid substitutions in the active site of *Bc* KWI-56 lipase. How different these pathways are will remain unanswered until the individual methods are tested in detail on the same protein and substrate.

5. Conclusions

The engineering of CAL-B by rational design and directed molecular evolution is still in its early stages. The extension of the current mutagenesis and shuffling procedures, as well as the application of alternative engineering methods promises new and improved hybrid biocatalysts. In addition, the development and adaptation of screening and selection techniques will be crucial for evaluating a larger, statistically significant portion of progeny.

More diverse protein libraries and higher throughput alone will not necessarily explain the fundamental aspects of lipase function. As suggested by Bocola

et al.,[37] the coordinated investigation of selected library members through experimental techniques, computational studies, and structure determination will be necessary to rationalize the observed changes on the molecular level. Such concerted efforts will help to address the controversy surrounding the effectiveness of a mutation in relation to its distance from the active site. Furthermore, these studies can test the feasibility of mechanistic features in proteins such as the proposed steric relays. Interestingly, observations underlining the importance of protein flexibility seem to be a reoccurring theme for lipases. While the fundamental importance of protein dynamics to catalysis is of little doubt, few studies have addressed this issue in wild-type enzymes and even fewer in engineered proteins. Ultimately, the sum of these individual aspects will form the basis for the more effective tailoring of CAL-B as a biocatalyst with the desired substrate specificity and enantioselectivity.

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