Communication

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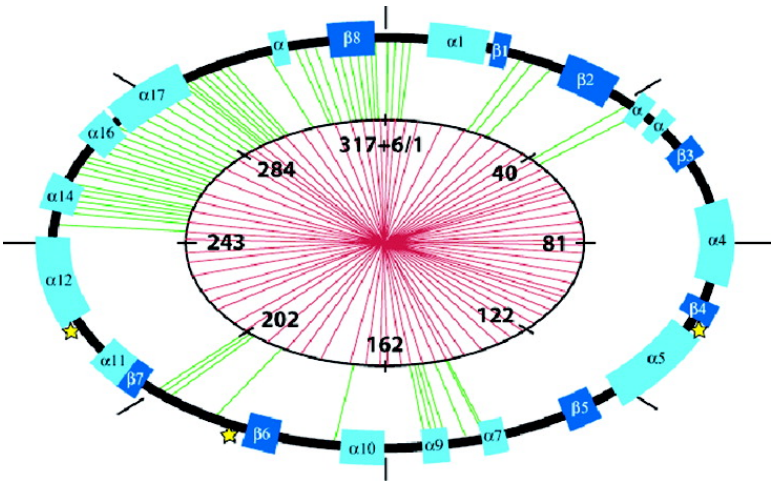

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Improving the Catalytic Activity of *Candida antarctica* Lipase B by Circular Permutation

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Lipases (EC 3.1.1.3) play an important role in asymmetric biocatalysis. Their broad substrate specificity, generally high regio- and enantioselectivity, as well as their ability to function in aqueous and organic reaction medium make them versatile tools for the kinetic resolution, derivatization, and polymerization of esters.[1−5] Suitable enzymes for a particular substrate can be identified by screening natural lipases or can be tailored by protein engineering. In the latter case, rational protein design, random mutagenesis, and DNA shuffling have generated laboratory catalysts with altered specificity, selectivity, and stability.[6−9] However, very few natural and lab-made lipases show activity and enantioselectivity for bulky substrates, such as esters of large secondary and tertiary alcohols.[10−14] It has been hypothesized that the cause for the poor turnover of these substrates arises from steric constraints in the lipase active site, yet protein engineers have so far failed to generate improved biocatalysts. Rather than substituting amino acids, we have speculated that structural constraints in lipases could be relaxed through protein backbone cleavage. Specifically, we hypothesize that the internal relocation of a protein's N- and C-termini in or near the active site can increase chain flexibility and active site accessibility, which could translate into higher activity for structurally more demanding substrates. Thus, we employed circular permutation to explore the effects of termini relocation on a lipase's catalytic performance. Working with the lipase B from *Candida antarctica* (CALB), we show that circular permutation of this enzyme results in numerous functional variants with improved catalytic activity.

Given the difficulty of identifying suitable permutation sites by rational design, we generated a comprehensive, combinatorial library of randomly permuted CALB variants. Starting with the wild-type CALB gene, we first introduced flanking oligonucleotide sequences which encode for the flexible six-amino acid linker (−GGTSGG−) to bridge the ∼17 Å distance between the original termini. After intramolecular ligation, the circular DNA was linearized in random positions using DNaseI.[15−17] Reaction conditions were chosen such that, on average, only a single cut per DNA strand was introduced. The resulting library of CALB permutants was then cloned into pPIC9 and transformed into *Pichia pastoris* GS115 for protein expression.[18] DNA sequence analysis of 96 randomly chosen members in the naïve library (∼5×10^5 colonies) confirmed the unbiased distribution of new termini over the entire length of the protein sequence (Figure 1). Next, functional variants in the CALB library were identified by colony screening on tributyrin plates. The DNA sequence analysis of functional members identified 63 unique protein sequences with termini in positions other than wild type (Figure 1).

The data indicate that CALB tolerates permutations in numerous positions over the entire length of the protein. When mapped on the wild-type CALB structure, the new termini not only coincide with surface loops but also interrupt secondary structure elements on the enzyme's surface and interior regions (Figure 2). Most noticeable is the concentration of functional permutations from amino acids 243 to 317. This sequence is largely surface-exposed, wrapping around the front of the α/β-hydrolase core and forming the alcohol-binding portion of the active site pocket (α17).[19,20] Two additional regions tolerant to permutation stand out: first, amino acids 44 and 47, which are located in close proximity to the oxyanion stabilizing residues; second, a cluster of permutations in α7/9 (amino acids 135−155), which constitutes the enzyme's lid region. We also identified two protein segments (residues 48−143 and 204−246) with no functional permutation. These regions make up the core of the α/β-hydrolase fold and include residues S105 and H224 of the catalytic triad. We speculate that the absence of functional permutation near these residues, as well as the presence of only a single site proximal to the triad's third amino acid (D187), reflects this region's importance to catalysis and possibly its relevance to protein folding.

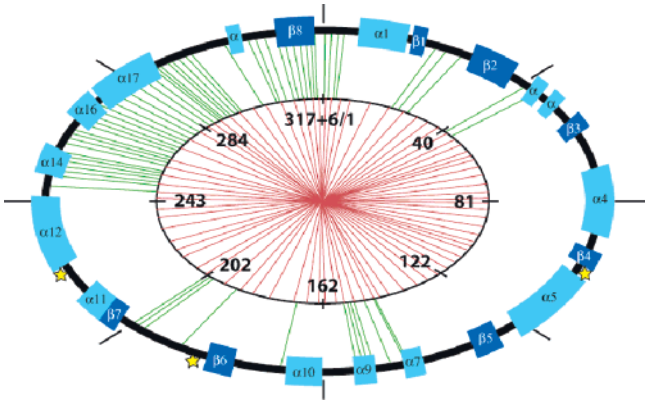

Figure 1. Circular permutation of CALB. Unbiased distribution of permutation sites over the entire length of CALB (317 amino acids) and the six amino acids linker in the native library (inner circle, red lines) and permutation sites of variants selected for tributyrin hydrolysis (outer circle, green lines). Secondary structures are shown in shades of blue; the three active site residues are marked by yellow stars.

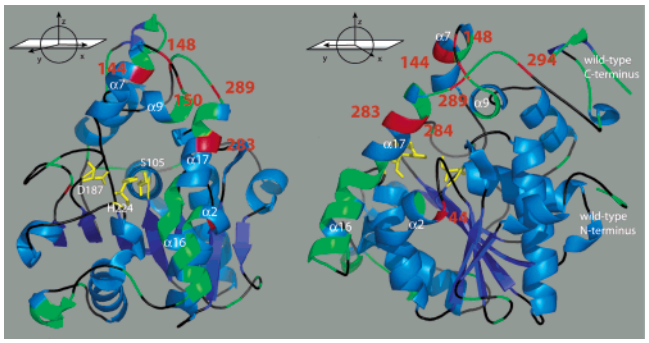

Figure 2. Front and side view of wild-type CALB (1tca).[19] The locations of permissible permutation sites are indicated in green, and candidates selected for subsequent in vitro characterization are labeled in red. The residues of the catalytic triad are shown in yellow.

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Table 1. Apparent Kinetic Constants for Selected CALB Variants with *p*-Nitrophenol butyrate and DiFMU Octanoate as Substrates

enzyme variants		*p*-nitrophenol butyrate				DiFMU octanoate			
name[a]	sequence[b]	K_M (μM)	k_{cat} (min^{-1})	k_{cat}/K_M ($min^{-1}\,\mu M^{-1}$)	relative specificity[c]	K_M (μM)	k_{cat} (min^{-1})	k_{cat}/K_M ($min^{-1}\,\mu M^{-1}$)	relative specificity[c]
wild type	L1/P317	410 ± 40	305 ± 10	0.74	1.0	2.6 ± 0.3	2 ± 0.1	0.8	1.0
cp44	G44/T43	690 ± 90	6 ± 0.5	0.01	0.01	5.6 ± 0.8	0.5 ± 0.05	0.1	0.13
cp144	L144/A141	550 ± 50	178 ± 7	0.32	0.4	2.0 ± 0.5	1 ± 0.1	0.5	0.6
cp148	A148/L147	500 ± 30	171 ± 4	0.34	0.5	3.5 ± 0.5	1.5 ± 0.2	0.35	0.4
cp150	S150/V149	510 ± 90	520 ± 45	1.02	1.4	2.7 ± 0.8	2.1 ± 0.2	0.8	1.0
cp283	A283/A283-KRPRINSP	280 ± 50	2971 ± 180	10.61	14.3	2.5 ± 0.5	25 ± 1.4	10.9	13.6
cp284	A284/A287-KRPRINSP	550 ± 70	2980 ± 200	5.42	7.3	8.8 ± 1.0	34 ± 4	3.8	4.8
cp289	P289/A284-KRPRINSP	260 ± 30	3258 ± 215	12.53	16.9	5.5 ± 1.0	120 ± 7	23	28.8
cp294	E294/A283	310 ± 40	73 ± 4	0.23	0.3	9.5 ± 2.0	2.6 ± 0.34	0.3	0.4

[a] CALB nomenclature: cp44 = circular permutated protein whose N-terminus starts at amino acid 44 of the wild-type sequence. [b] N- and C-terminal amino acids (all in single-letter code) are listed. Small variations in chain length of individual permutants are caused by reading frame shifts and staggered ends upon DNaseI digestion. [c] Relative specificity = k_{cat}/K_M (variant)/k_{cat}/K_M (wild type).

To examine the impact of circular permutation on catalysis, we selected eight functional CALB variants with termini in or near the active site for detailed kinetic characterization (Figure 2). Following overexpression in *P. pastoris*, the proteins were purified to homogeneity, and kinetic data for these variants were measured on two standard lipase substrates, *p*-nitrophenol butyrate (*p*NB) and 6.8-difluoro-4-methylumbelliferyl (DiFMU) octanoate (Table 1).[18]

Our kinetic analysis confirmed that circular permutation has a significant impact on CALB's catalytic performance. The most substantial improvements in enzymatic activity over wild-type CALB were observed upon termini relocation into α17. Three of the four variants (cp283, cp284, cp289) show a consistent 10-fold improvement in their apparent k_{cat} values for *p*NB and up to 60-fold increases in DiFMU octanoate turnover. Removal of the C-terminal peptide extension, an engineering artifact found in all three variants, left catalytic rates unchanged (data not shown). The impact of backbone cleavage in α17 on active site accessibility and the rate-limiting deacylation step in wild-type CALB are under investigation. In contrast, a removal of the entire protein fragment (amino acids 284−293) in cp294 proves detrimental to catalysis. Whether the deletion dismantles the active site pocket, preventing productive substrate binding, or affects protein stability as the disulfide bond-forming C293 is eliminated remains unclear.

The backbone cleavage in the lid region (cp144, cp148, cp150) showed only moderate effects on hydrolysis of our test substrates. Both K_M and k_{cat} for all three variants stay within 2-fold of the parent enzyme under the described assay conditions. Structure models predict close interactions of this protein region with the substrate's acyl portion.[19] Thus, future experiments may explore the turnover of bulkier carboxylates by these CALB variants. Furthermore, circular permutation of the lid region may alter the enzyme's response to changes in the reaction medium. The latter can affect lipase activity by modulating conformational changes in the lid region.

Finally, the kinetic data for cp44 show a 10−100-fold reduction in relative specificity, compared to that of wild-type CALB. The close proximity of the permutation site to the oxyanion binding pocket likely results in the topological misalignment of the active site residues. Consistent with our observation of permutation-free protein segments, we hypothesize that protein permutation does increase local backbone flexibility. While such flexibility seems detrimental at positions in proximity to active site residues, the relaxation effects can be beneficial when applied to protein regions which contribute to the active site topology but do not directly carry a side chain involved in catalysis.

In summary, CALB engineering by circular permutation has generated 63 new, unnatural lipase variants. Kinetic analysis confirmed that these protein variants can have sustained or improved catalytic function on multiple substrates over wild-type, mutant, and shuffled CALBs.[8,9,21] The observed rate enhancements are believed to result from improved active site accessibility and increased local protein backbone flexibility.

Beyond exploring the substrate specificity and enantioselectivity of these lipase variants, future studies will need to address the impact of permutations on CALB's structural integrity and stability on the molecular level. Furthermore, mutagenesis of selected CALB permutants may help to fine-tune their catalytic activities. Finally, the concept of circular permutation could prove valuable for tailoring other lipases and hydrolases in general.

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Supporting Information Available: Full experimental details (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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