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Enantioselective Enzymes by Computational Design and In Silico Screening**

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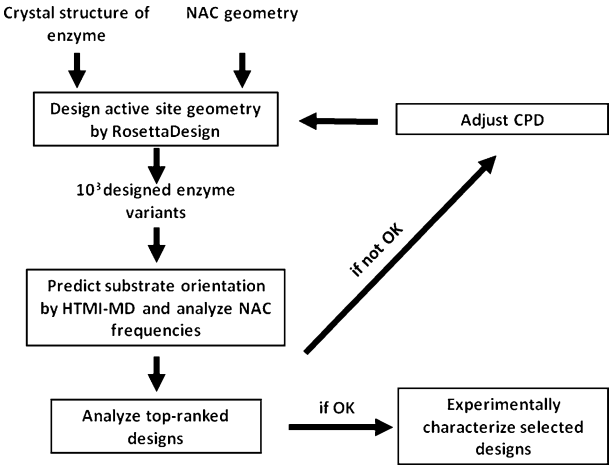
Abstract: *Computational enzyme design holds great promise for providing new biocatalysts for synthetic chemistry. A strategy to design small mutant libraries of complementary enantioselective epoxide hydrolase variants for the production of highly enantioenriched (S,S)-diols and (R,R)-diols is developed. Key features of this strategy (CASCO, catalytic selectivity by computational design) are the design of mutations that favor binding of the substrate in a predefined orientation, the introduction of steric hindrance to prevent unwanted substrate binding modes, and ranking of designs by high-throughput molecular dynamics simulations. Using this strategy we obtained highly stereoselective mutants of limonene epoxide hydrolase after experimental screening of only 37 variants. The results indicate that computational methods can replace a substantial amount of laboratory work when developing enantioselective enzymes.*

Recent successes with de novo computational protein design (CPD)[1] and redesign of enzyme active sites[2] suggest that computational methods can be used for controlling enzyme selectivity, which is an important goal in industrial biocatalyst development. Enantioselectivity engineering is often pursued by directed evolution, in most cases with the use of large mutant libraries.[3] Although important successes have been reported,[3] further development could benefit from improved predictability of mutant properties and a higher abundance of beneficial substitutions in mutant libraries. We have recently explored the use of computational methods for the design of small mutant libraries harboring thermostable enzyme variants.[4] In the work reported herein, we demonstrate the use of computational library design for engineering enzyme stereoselectivity.

The computational strategy that we explored is to place a substrate in the enzyme active site in an orientation required for the desired enantioselective conversion and to redesign the active site to stabilize that geometry. Protein sequences forming predefined substrate-binding sites or sites with shape complementarity to transition-state models can be generated by CPD methods such as implemented in RosettaDesign[5] after which laboratory screening is carried out to identify improved variants.[6] In the approach that we termed CASCO (Scheme 1), high-throughput molecular dynamics simulations



Scheme 1. The developed CASCO framework for redesign of catalytic selectivity by integration of computational enzyme design and HTMI-MD. The computational design includes the introduction of steric hindrance to prevent unwanted substrate binding modes.

that predict relative reactivity and thereby enantioselectivity replace most of the experimental screening. The strategy was tested with a challenging case of biocatalytic relevance: the design of a pair of enantiocomplementary epoxide hydrolases for the enantioselective transformation of cyclopentene oxide (**1a**) to yield either (*R*,*R*)- or (*S*,*S*)-cyclopentane-1,2-diol (**1b**) (Scheme 2). As the stereochemical outcome of this asymmetric conversion is controlled by regioselectivity of water attack, accurate placement of the substrate in the active site is essential. This is highly challenging because epoxide **1a** lacks bulky or polar groups that could position the substrate in a defined way by directional interactions such as hydrogen bonds or electrostatic attraction.

Asymmetric enzymatic synthesis of diol **1b** requires an active site geometry that binds substrate **1a** in a reactive

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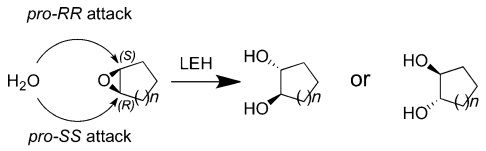


1a (*n*=1) (*R*,*R*)-**1b** (*S*,*S*)-**1b**
2a (*n*=2) (*R*,*R*)-**2b** (*S*,*S*)-**2b**
3a (*n*=3) (*R*,*R*)-**3b** (*S*,*S*)-**3b**

Scheme 2. Hydrolyis of meso epoxides by limonene epoxide hydrolase (LEH). Since the reaction occurs with inversion of configuration at the attacked carbon atom, the stereochemical outcome is governed by regioselectivity of water attack.

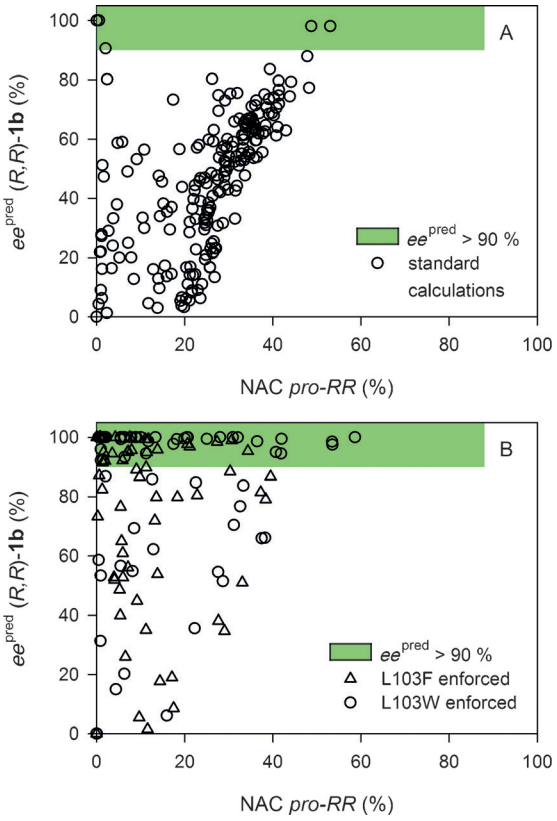


Figure 1. HTMI-MD-based prediction of NACs and enantiomeric excess of variants designed for pro-*RR* attack. The predicted enantiomeric excess (ee^{pred}) was calculated using Equation S2 (Supporting Information). Each symbol represents one computationally designed variant with an ee^{pred} of more than zero. Additional examples can be found in the Supporting Information, Figures S3 and S4. A) Variants for pro-*RR* attack obtained by computational design without enforcing selective steric hindrance. B) Variants designed for selective pro-*RR* attack by introduction of a Phe or Trp at position 103.

mode for either pro-*RR*[7] or pro-*SS* hydrolysis.[3f,8] Therefore, the design procedure aimed to identify a set of mutations that create an active site cavity in which the substrate is positioned selectively in one of the two catalytic orientations. The diversity used as input for the computations was generated by simultaneous introduction of substitutions at eleven positions around the active site (M32, L35, L74, M78, I80, V83, L103, L114, I116, F134, F139) to any of the nine hydrophobic residues (AFGILMPVW). The substrate was docked in the crystal structure of limonene epoxide hydrolase either in a pro-*RR* or a pro-*SS* attack position and sequence-conformational space was searched by RosettaDesign[5d] to discover substrate-bound structures with a low energy. These should correspond to variants that have a three-dimensional structure that is correctly formed in vitro and that possess an active site with shape complementarity to substrate bound in the desired orientation.[5e,9] The methods produced 236 initial pro-*SS* designs and 230 pro-*RR* designs (Supporting Information, Table S1).

CPD methods that search for variants with a low-energy structure may generate mutants that besides the required substrate-binding modes also allow undesired substrate poses,[1b,10] thus contaminating designed libraries with ineffective mutants. To reduce their occurrence, we included in the CASCO approach two rounds of computational screening by HTMI-MD (high-throughput–multiple independent MD simulations), a technique to increase the conformational space sampled by MD simulations (Supporting Information, Table S1).[11] This allowed ranking of the designed enzyme variants at low computational cost. Using these MD simulations, the reactivity and selectivity with respect to pro-*RR* or pro-*SS* attack[11] were predicted. As a measure of reactivity, the fraction of time that the enzyme–substrate complex spends in a pro-*RR* or pro-*SS* attack conformation was quantified by using geometric criteria for near attack conformations (NACs), which are conformations that approach the transition state structure (Supporting Information, Scheme S1).[12] The ratio of the NAC frequencies for enantiomers was used to predict enzyme enantioselectivity.[11,13] The criteria for pro-*RR* and pro-*SS* attack (Supporting Information, Scheme S1) were defined on the basis of published quantum mechanical modeling.[8b,14] Briefly, NACs were defined as conformations in which the distances between the reacting atoms are smaller than the van der Waals contact distances, angles between reacting atoms deviate less than 20° from those in the transition state, and all hydrogen bonds that stabilize the transition state are present.[10–12] It has been shown that small differences in the geometric definition of NACs have negligible effects when NACs are used to predict the relative catalytic rates for two different states.[12]

The MD simulations predicted that the initial designs indeed should catalyze the desired reaction, but without displaying the exclusive pro-*RR* or pro-*SS* regioselectivity required for enantioselective product formation (Figure 1 A). Inspection of MD trajectories of the designed mutants indicated that there was too much space around the (1*R*,2*S*)-cyclopentene oxide docked in reactive poses, which enabled the substrate to move both into pro-*RR* and pro-*SS* orientations in these initial designs.

To obtain the required selectivity, we introduced steric hindrance that should block such undesired substrate-binding modes. For this, we analyzed the effect of introducing bulky residues (W, F, and also Y if the original residue was an F) at each of the 11 target positions lining the active site. This procedure indicated that the mutations L103F/W, L114F/W,

I116F/W, and F134W/Y could sterically hinder pro-*RR* attack, while the mutations L35F/W, L74F/W, M78F/W, I80F/W, and L103F/W could prevent pro-*SS* attack. The forced introduction of these mutations was necessary, as these bulky substitutions only occurred in low frequencies amongst the initial designs (Supporting Information, Figure S1). Algorithms to automatically carry out such tasks are still under development, and will aid future design projects.[15] After selecting a substitution that enforces steric hindrance, the other ten residues were computationally redesigned in separate design calculations, which resulted in 945 pro-*RR* and 931 pro-*SS* designs with improved sequence diversity (Supporting Information, Figures S1, S2, Table S1).

When these second-generation designs with enforced steric hindrance were subjected to HTMI-MD simulations, a much larger fraction of the variants exhibited the desired enantioselectivity (Figure 1B; Supporting Information, Figures S3, S4). Of the initial designs, 1.6% had an ee^{pred} of over 90%, whereas after the introduction of steric hindrance by mutations L103F or L103W, over 20% of the designs was calculated to have a high ee^{pred} for pro-*RR* attack (Supporting Information, Table S1). Similarly, substitutions L35F/W, M78W, and I80F/W increased the fraction of the enantiocomplementary designs with an ee^{pred} for pro-*SS* attack from 10 to 20% of the designs (Supporting Information, Table S1).

To evaluate the design, we expressed and tested the 10 designs with the highest predicted pro-*RR* selectivity as well as 27 pro-*SS* designs. For this a recently engineered thermostable variant of LEH was used[4b] because thermostability increases the probability that mutations are tolerated without formation of misfolded protein.[16] Conversion assays with the purified mutant enzymes with analysis of diol produced from cyclopentene oxide **1a** by chiral GC revealed that 28 of the 33 active variants had the enantioselectivity they were designed for (Supporting Information, Table S2). Of these, nine were highly selective, producing the desired diol with an *ee* of over 75%. The most enantioselective variants were pro-RR-8 which contains five mutations and produces (*R*,*R*)-**1b** with an *ee* of 85% and pro-SS-16, which has seven mutations and forms (*S*,*S*)-**1b** with 90% *ee* (Figures 2 and 3; Supporting Information, Figure S5). Both these variants have a bulky residue (L103F and L35W, respectively) that was introduced to prevent a substrate binding mode that would allow nucleophilic attack by water at the unwanted position, and the *ee* value demonstrates that in these variants 92% and 94%, respectively, of the nucleophilic attack events occurred on the correct carbon atom (see the Supporting Information, Equation S2). These results show that there is a good correlation between the predicted and the experimentally observed enantioselectivities.

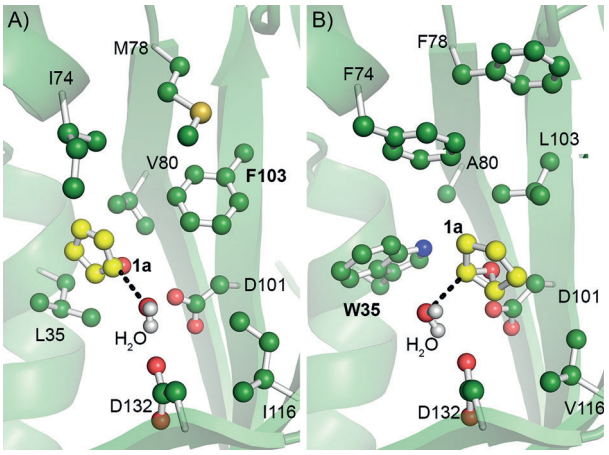


Figure 2. Predicted structures of the active sites of limonene epoxide hydrolase variants pro-RR-8 (A) and pro-SS-16 (B). The docked substrate **1a** is shown in yellow. The water molecule performing the nucleophilic attack is also shown. Residues that were introduced to prevent substrate binding in the undesired pose are labeled in bold.

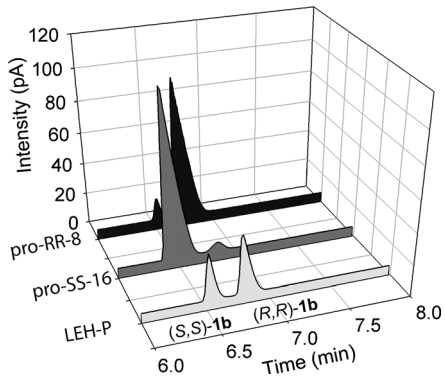


Figure 3. Chiral-phase gas chromatography of diols **1b** produced by LEH variants. The plot displays FID detector signal intensity versus the retention time on a Hydrodex b-TBDAc column (Aurora Borealis, The Netherlands).

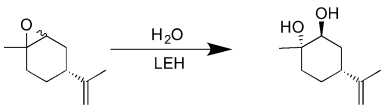
The two most selective variants (pro-RR-8 and pro-SS-16) were characterized in more detail. Both variants were expressed at 50 mg protein per liter of culture broth, which is comparable to the yield of the parent LEH-P.[4b] The two variants maintained good catalytic activity for the hydrolysis of **1a**, albeit with lower rates than variant LEH-P (Table 1).

Table 1: Computationally predicted and experimentally observed enantioselectivities of the best limonene epoxide hydrolase variants for the hydrolysis of cyclopentene oxide (**1a**).[a]

	Computational predictions			Experimental analysis		
Variant	Active site mutations[a]	NAC pro-*RR* [%]	NAC pro-*SS* [%]	k_{obs} [s^{-1}]	Major product	*ee* [%]
WT (LEH-P)	none			0.026 ± 0.0006	(*R*,*R*)-**1b**	23.9
pro-*RR*-8	M32L/L74I/I80V/**L103F**/F139L	5.95	0.004	0.0048 ± 0.0003	(*R*,*R*)-**1b**	85.5
pro-*SS*-16	M32L/**L35W**/L74F/M78F/I80A/I116V/F139L	0.69	48.1	0.0031 ± 0.0003	(*S*,*S*)-**1b**	90.2

[a] NAC percentages obtained from 10 independently initialized 100 ps MD simulations. The mutations enforcing steric hindrance are in bold. Data on the other improved variants are given in the Supporting Information, Table S2.

Variants pro-RR-8 and pro-SS-16 had apparent melting temperatures of 69.5 °C and 66.5 °C, respectively, compared to 68.0 °C for LEH-P, showing that the thermostability which was introduced earlier was maintained (Supporting Information, Table S3). Variants pro-RR-8-and pro-SS-16 exhibited also enantioselectivity with the larger alicyclic epoxides **2a** and **3a** (Scheme 2). For **2** and **3**, pro-RR-8 was moderately (*R*,*R*) selective while pro-SS-16 was highly (*S*,*S*)-selective (Supporting Information, Table S3). The natural substrate limonene epoxide **4a** (Scheme 3) was also converted with high enantioselectivity (Supporting Information, Table S3).



rac-**4a** (1*R*,2*S*,4*R*)-**4b**

Scheme 3. Hydrolysis of limonene epoxide *rac*-**4a** by LEH.

The enantioselectivity of mutants pro-RR-8 and pro-SS-16 is similar to that of two variants that were discovered by directed evolution;[3f] mutants H173 and H178 produced (*R*,*R*)- and (*S*,*S*)-cyclopentanediol with an *ee* of 80% and 93%, respectively. The variants pro-RR-8 and pro-SS-16 each have only one substitution in common with the corresponding directed evolution mutants (L74I in pro-RR-8 and H173, I116V in pro-SS-16 and H178). These particular substitutions do not have a large influence on the shape of the active site (Figure 2). Whereas the outcomes of directed evolution and computational approach are similar in terms of enzyme performance, they differ in the sequences of the enzyme that are produced. More importantly, the approaches differ in experimental screening effort, which encompassed 4700 variants examined by chiral GC for directed evolution[3f] but only 37 mutants in case of the CASCO-based library described herein. Thus, computational library design and computational screening can replace most of the laboratory screening of a directed evolution project.

In conclusion, we developed a strategy for redesign of catalytic selectivity by computation (Scheme 1). The combined use of computational design and molecular dynamics screening resulted in the rapid discovery of epoxide hydrolases that produced diols with high enantiomeric excess. We anticipate that computational library design will increase reliability and cost efficiency of protein engineering efforts aimed at controlling product profiles of enzymes relevant for applied biocatalysis.

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