Tunnel engineering enables multifaceted improvements in halogenase

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In a recent article in *Nature Catalysis*, Prakinee et al. applied a mechanism-guided protein-engineering strategy to optimize the behaviors of a flavin-dependent halogenase by tuning the hydrophobic interactions within the tunnel for hypohalous acid (HOX) transport. This strategy allows the control of the fate of enzymatic intermediates at low cost.

Biocatalysis is one of the most promising types of catalysis in the 21st century, primarily because biosystems are capable of catalyzing a large number of reactions that are otherwise hard to achieve through other types of catalysis.[1] The unique reactivities of biosystems mostly originate from the enzymes where the microenvironment of the active site has evolved over a long time to meet the natural needs of their biological hosts.[2] Directed evolution showcases how these natural behaviors of a given biosystem can be fine-tuned at multiple levels to boost the biosystem's natural behavior and even create new behaviors, greatly speeding up the process of transforming efficient biocatalytic research into industrial applications.[3] It is encouraging to witness how the rapid development of technologies has changed the way that scientists study biocatalysis, including but not limited to easier access to protein structures and identification of protein-substrate interactions,[4] less time-consuming experimental construction and screening of biosystems,[5] and direct observations of biological intermediates.[6] These developments in technology can significantly help scientists better understand biocatalysis, thereby bringing more opportunities to the discovery of useful biocatalysis. Recently, Prakinee et al. reported a semi-rational protein-engineering strategy that integrates mechanistic investigations, high-throughput screening, and computational modeling. They used this strategy to unveil and solve the problem of the flavin-dependent halogenase Thal by identifying, mechanistically studying, and engineering Thal's HOX-transport tunnel for improved reactivity, thermal stability, pH tolerance, and substrate scope with a single mutation.[7]

The catalytic cycle of Thal comprises five key steps (Figure 1). First, Thal accepts reduced flavin ($FADH^-$) generated from a flavin reductase to produce Thal:$FADH^-$. Second, this highly oxidative Thal:$FAD_{C4a\text{-}OOH}$ reacts with one adjacent halide anion (X^-) to produce the hypohalous acid (HOX) and Thal:$FAD_{C4a\text{-}OH}$. Third, the HOX moves to the active site around 10 Å away from the flavin-binding site of Thal through a tunnel. Fourth, the HOX reacts with tryptophan to produce the C6-halogenated product and water. Fifth, Thal:$FAD_{C4a\text{-}OH}$ reoxidizes to FAD and releases one water molecule, regenerating Thal and completing the catalytic cycle. There are two side reactions in this system: one is the releasing of hydrogen peroxide from Thal:$FAD_{C4a\text{-}OOH}$ to produce FAD before the formation of HOX, and the other is the HOX leakage from the tunnel before HOX reaches the active site to react with tryptophan. A previous study demonstrated that the HOX leakage is one important factor that accounts for the low catalytic efficiency of Thal.[8]

On the basis of this knowledge, the authors identified six possible tunnels for the transport of HOX by modeling the empty spaces that start from the active site and end at the surface of Thal, where they created the empty spaces by connecting a series of spheres surrounded by amino acid residues with a minimum radius of 1 Å. The tunnel that connects the flavin-binding site and the tryptophan-binding site of Thal was chosen as the most relevant one. Tunnel analysis and docking results of HOX at different positions in the tunnel indicated three bottlenecks around the flavin gate, HOX gate, and tryptophan gate. The authors chose only the HOX gate and the tryptophan gate for protein engineering to avoid perturbance on the formation of flavin intermediates. In addition, molecular dynamics (MD) simulation revealed that the HOX gate remained the bottleneck of the tunnel at different protein dynamics, suggesting that it is the key part in HOX transport. Site-saturation mutagenesis on the chosen amino acid residues in the HOX gate led to Thal:V82I. Compared with Thal:WT (wild type), Thal:V82I showed 50% less HOBr leakage, a 12°C higher protein melting point, a 5-fold greater reaction yield at 45°C, and a 2-fold greater reaction yield at neutral to basic pH while maintaining the WT's original selectivity in the bromination of tryptophan.

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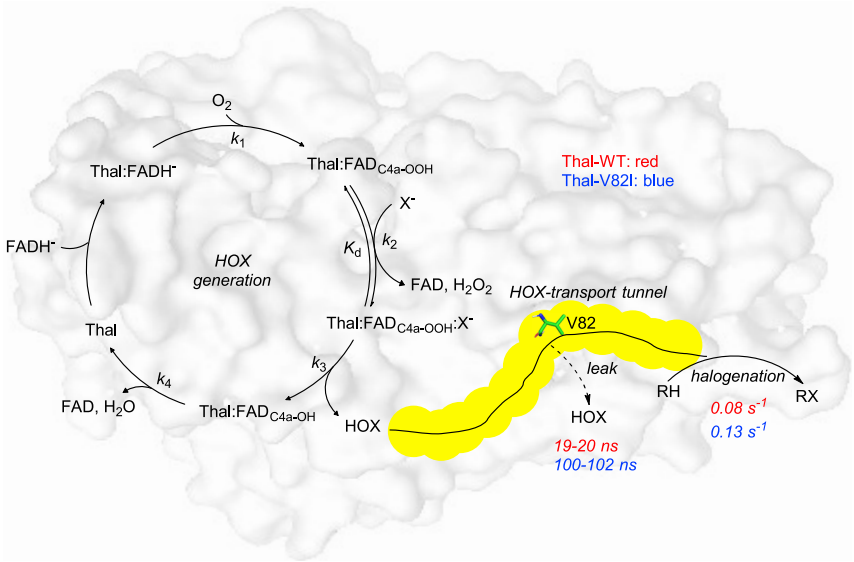

Figure 1. Mechanism of Thal-catalyzed halogenation

Times and rate constants are labeled red for Thal:WT and blue for Thal:V82I. WT, wild type; RH, tryptophan; RX, 6-halogenated tryptophan; yellow contour, HOX-transport tunnel; V82, the key amino acid residue at the HOX gate of Thal:WT.

To understand the leaking process during the reaction, the authors performed MD simulations on the dynamics of the HOBr-protein complex during the course of the first 1,000 ns of the reaction, where they characterized the extent of leakage by analyzing the distances between HOBr and E358, an amino acid residue at the HOX gate. This distance stayed at around 10 Å (in tunnel) at the early stage of the reaction and jumped to around 60 Å (out of tunnel) after a certain period of time, which was 19–20 ns for Thal:WT and 100–102 ns for Thal:V82I. In addition, the water network within the tunnel of Thal:V82I was less occupied than that within the tunnel of Thal:WT, suggesting a smaller chance of HOX leakage given that hydrophobic interaction plays an important role in the transport of polar molecules inside a protein.

The authors then performed a series of experimental studies to understand the kinetics of both enzymes (Figure 1). First, the steady-state bromination progressed faster during the course of the reaction for Thal:V82I (K_m = 21.4 μM) than for Thal:WT (K_m = 76.0 μM) in the presence of the NADH regeneration system despite the fact that Thal:V82I and Thal:WT have similar rate constants at around 2.7 min^{-1} upon the completion of the overall reaction. Afterward, the authors compared the kinetics of the key steps in the catalytic cycle (Figure 1) by correlating the absorbance or fluorescence of different flavin species at their signature wavelengths for $FADH^-$, $FAD_{C4a\text{-}OOH}$, $FAD_{C4a\text{-}OH}$, and FAD together with the production of brominated tryptophan at 0.001–100 s between the single-turnover reactions catalyzed by Thal:V82I and Thal:WT. The authors observed that both enzymes had similar rate constants in the key steps of $FADH^-$ oxidation, HOX production, and the side process of H_2O_2 dissociation. The main difference lay in the FAD regeneration step, which is irrelevant to the single-turnover experiment but has been shown in previous studies to be rate limiting for many other flavin-dependent monooxygenases.[9] These results suggest that the improved reactivity mainly comes from the decreased amount of HOX leakage. Furthermore, the authors demonstrated that Thal:V82I bears a wider substrate scope than Thal:WT as a result of the conformational change in the active site, resulting in the differences in the binding mode of substrates and dictating new reactivities.

In summary, Prakinee et al. have investigated HOX transport in the flavin-dependent halogenase Thal to help discover a single mutant, Thal:V82I, that has significantly improved reactivity and stability and broadened substrate specificity while maintaining the original selectivity of the WT. Mechanistic studies reveal that the HOX leakage is dramatically minimized in the designed mutant compared with the WT while the rate constants of other key steps in the catalytic cycle remain on the same timescales as those of the WT. We anticipate that this mechanism-guided protein-engineering strategy will inspire further research focusing on controlling the dynamics of enzymatic intermediates for the discovery of novel reactivities.

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DECLARATION OF INTERESTS

The authors declare no competing interests.

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A rational approach to re-engineer the catalytic efficiency of flavin-dependent halogenases

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Recently in *Nature Catalysis*, Prakinee et al. used a novel approach where they re-engineered the hypohalous acid (or HOX) intermediate transfer tunnel of a tryptophan 6-halogenase enzyme to create a catalytically robust and highly efficient variant enzyme.

A suitably positioned carbon-halogen moiety is known to improve the molecular efficacy, potency, biophysical, and pharmacokinetic properties of many bioactive molecules and pharmaceutical drug compounds compared with non-halogenated analogs. As a direct consequence, the last few decades have witnessed an increase in the identification of halogenated secondary metabolites and natural products. Most importantly, the halogenase enzymes responsible for halogenation in these biosynthetic gene clusters have gained wider interest because of the huge biotechnological potential of the regio- and site-specific halogenation of advanced pharmaceutical intermediates and synthetic precursors of fine chemicals.[1–3] Site-selective halogenation using halogenase enzymes followed by a metal-catalyzed cross-coupling reaction—such as Suzuki-Miyaura and Heck reactions—could also offer a new type of functionalization of carbon-halogen bonds and could aid the synthesis of novel molecular scaffolds.[2]

Among the four main types of cofactor-based halogenase enzyme classes, flavin-dependent halogenases (Fl-Hals) are the most promising candidates for the above-mentioned applications and for natural and non-natural biosynthetic pathway and chemo-enzymatic reactions.[4] In native microorganisms, Fl-Hals are non-essential for survival and growth, indicating a lower level of evolutionary pressure to catalyze reactions with ease and efficiency, a logical consensus for their very own sluggish nature. Being almost 1,500 times slower than any exemplified industrial enzymes (e.g., tryptophan synthases), Fl-Hals also undergo a mechanism-based inhibition and inactivation, further limiting their turnover and rate of substrate uptake. Several targeted (site-specific) and random mutagenesis approaches have been reported on Fl-Hals over the years to increase their synthetic repertoire and to address this inherent inactivity. Although such methods as creating combinatorial libraries, cross-linking Fl-Hals for enzyme aggregate (CLEA) preparation, substrate walking, and family-wide activity profiling with the support of high-throughput screening assays provide moderate improvements, the required targets for direct industrial applications have yet to be achieved.[5,6]

As recently reported in *Nature Catalysis*, Prakinee et al. have adapted a completely different mechanism-based approach. Here, the authors attempted to re-engineer the hypohalous acid (or HOX) intermediate transfer tunnel in a selected tryptophan 6-halogenase enzyme (Thal) to improve the catalytic efficiency.[7] In Fl-Hals, the substrate

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