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Sweetly Expanding Enzymatic Glycodiversification

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Directed evolution is a powerful tool to modify substrate specificity for a wide array of enzyme catalysts. In this issue of *Chemistry & Biology*, Thorson and coworkers use directed evolution to increase the catalytic proficiency of a model glycosyltransferase, OleD, 300-fold for a nonphysiological substrate (Williams et al., 2008).

Carbohydrates are responsible for mediating diverse biological processes and developing carbohydrates toward controlling these processes will likely have a significant medicinal impact. The chemistry of carbohydrates is, however, formidable. Enzymatic synthesis has been flaunted as one potential solution, but strategies to expand substrate specificity have tempered the use of glycosyltransferases (GTs), Nature's primary glycosylation catalysts. Scientists have developed a number of tools to generate altered protein catalysts that furnish synthetically useful molecules, yet applying these tools to enzymatic glycosylation has been remarkably tricky (Hancock et al., 2006).

The lack of widely applicable selection or screening strategies has been a primary obstacle. Screening strategies for glycosylation include the use of coupled assays—the product produced by glycosylation is a substrate for another enzyme that catalyzes the release of a chromophore from the glycosylated product. This has formed the basis for identifying improved glycosynthase variants, although it is limited by the substrate specificity and efficiency of the monitoring enzyme (Mayer et al., 2001; Kim et al., 2004). A selection approach for glycosynthases involves using chemical complementation and a yeast three-hybrid assay; however, this requires the dedicated synthesis of complementation substrates (Lin et al., 2004; Tao et al., 2008). A sialyltransferase has been screened using fluorescence-activated cell sorting (FACS) where the fluorescent product from the improved variant remains trapped within the cell (Aharoni et al., 2006), while fluorescence-based microtiter plate assays were used to identify improved variants of a natural product glucosidase (vide infra). Recently a screening approach monitoring the decrease of pH using bromothymol blue was reported for a blood group galactosyltransferase (Persson and Palcic, 2008), although glycosylation of the pH indicator may be a consideration in some cases. An alternative screening approach may involve lactate dehydrogenase and pyruvate kinase monitoring of UDP release, as a sugar is transferred from UDP onto an acceptor by a glycosyltransferase (Liu and Tanner, 2006).

Prior research in the Thorson group, aimed toward the development of new glycosylation catalysts, resulted in the development of a novel high-throughput screening methodology for a glycosyltransferase (Williams et al., 2007). The key step was a reduction in fluorescence of an acceptor molecule upon glycosylation by the GT. This screening methodology was applied to the directed evolution of OleD and enabled the researchers to switch the acceptor substrate specificity from the aminosugar moiety of oleandromycin toward 4-methyl umbelliferone, a fluorescent nonphysiological substrate. In addition, the breadth of donor substrates was also enlarged to accommodate 13 different UDP-sugars. Three amino acid substitutions were determined to be responsible for conferring the altered substrate specificity and improvement in OleD activity.

In the present article, the researchers further broaden the acceptor substrate specificity of OleD GT toward a nonfluorogenic substrate, novobiocic acid, by saturation mutagenesis at the specific amino acid positions identified through use of the previous high-throughput screen as being critical toward enhanced activity. The improved catalytic efficiency of the final OleD triple mutant also enabled transfer of an expanded repertoire of UDP-sugars leading to the identification of eight novel glycorandomized novobiocic acid-containing natural products, for which preparation by chemical synthesis would be a significant endeavor. This work clearly demonstrates the power of enzymatic glycosylation.

For this research, a high-throughput screen with novobiocic acid as the acceptor was not feasible, due to the lack of a spectroscopic change upon glycosylation; therefore, any screening process would be accomplished by HPLC, severely limiting the number of potential mutants that could be evaluated. Thus, the researchers hypothesized that exploring all variants at the critical residues responsible for expanding the promiscuity of OleD in the former study, would lead to progeny with improvements in the desired catalytic activity, and provide a library size (~300 colonies) amenable toward HPLC screening. Saturation mutagenesis (Williams et al., 2007) at each of the critical amino acid residues using OleD optimized in the fluorescence-based assay as template resulted in improved variants at two positions. Combinations of these improved variants culminated in a triple mutant with impressive 200- and 300-fold improvements in efficiency (k_{cat}/K_m) for UDP-Glc and novobiocic acid, respectively. Two of these mutations were proximal to the oleandromycin binding site, and one was in a loop proximal to the UDP-sugar binding site, as shown in Figure 1. This is an impressive study demonstrating a broader donor and acceptor substrate specificity for OleD than previously known, and the potential for saturation mutagenesis to evolve enzyme substrate specificity to catalyze a synthetically desirable transformation.

It would be interesting to ascertain whether the evolved variant remains as catalytically proficient as the wild-type

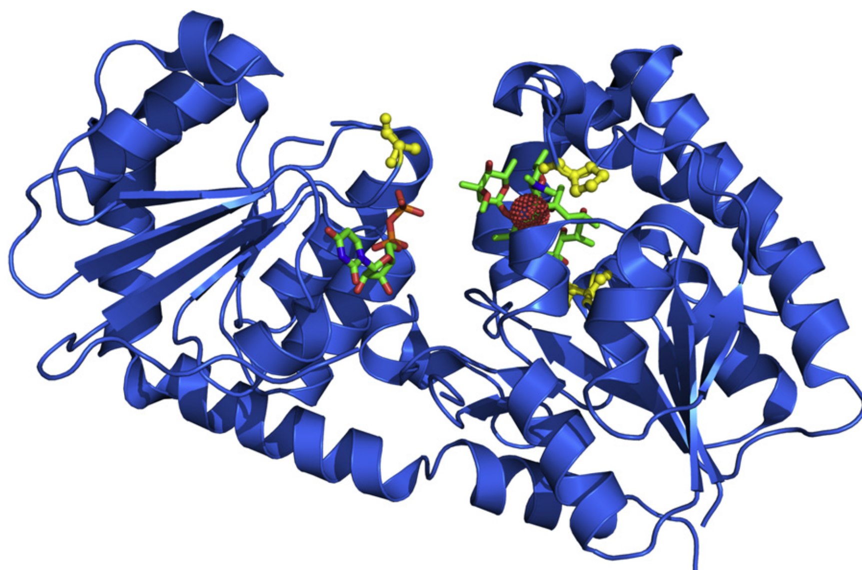

Figure 1. A Crystal Structure of Wild-Type OleD with Bound UDP and Erythromycin
The ball-and-stick residues in yellow correspond to the three "hot spots". The 2° hydroxyl functionality of desosamine is indicated as a dotted sphere. PDB code: 2IYF (Bolam et al., 2007). Figure was generated by PyMol (http://pymol.sourceforge.net).

enzyme in the physiological reaction and, additionally, how the significant structural differences between the two acceptors are accommodated in the active site. The significant electronic properties of the two hydroxyl functionalities may also be important to effect catalysis. The umbelliferone (pK_a ~8) versus the desosamine (pK_a ~15) functionalities may strongly influence the ability of the wild-type or evolved variants to activate each acceptor by general base catalysis.

There are a number of different approaches already available to screen or select for the evolution of glycosylation catalysts, and each method offers different opportunities and has its associated challenges or limitations. The GT-B fold of family 1 GTs (Bolam et al., 2007), where acceptors bind the N-terminal domain and donors bind the C-terminal domain, potentially enables the application of screening and selection tools to evaluate hybrid enzymes prepared by mixing domains from two different GTs. The research reported by Thorson and coworkers in this issue of *Chemistry & Biology* not only demonstrates the use of an evolved GT variant to accomplish diverse natural product glycosylation, but promotes the paradigm of exploring any identified "hot spots" by saturation mutagenesis to facilitate enzyme engineering. This may be of particular importance for enzyme systems where high-throughput or selection-based strategies have yet to be developed.

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