Biochemical, structural and dynamic investigation of transglycosylation in glycoside hydrolases for the chemoenzymatic synthesis of rare sugar-containing oligosaccharides

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1. Context and background

Despite considerable progresses in the field of synthetic glycochemistry, the practical and scalable access to carbohydrate structures is an extremely challenging and labour-intensive process that generally leads to limited overall yields. In the recent decades, driven by advances in the engineering of enzymes, biocatalytic methods have increasingly penetrated organic chemistry, providing complementary or alternative tools, overcoming some of the bottlenecks associated with classical chemical techniques and opening the way towards the synthesis of more complex sugar-based assembly. In this context, carbohydrate-active enzymes are attractive new tools for the chemist’s toolbox, since they operate in mild, aqueous conditions, are stereospecific (i.e. anomeric specificity) and highly regioselective, and limit or even obviate the protection/deprotection cycles that are used in classical glycochemistry.

In Nature, glycosyltransferases (GTs) are the major carbohydrate-active enzymes to target the formation of glycosidic bonds, but for in vitro synthesis of glycomotifs, glycan phosphorylases and mostly glycoside hydrolases (GHs), as well as glycosynthase mutants thereof, have also proved to be useful and often more accessible biocatalysts for the synthesis of tailor-made carbohydrate structures. This is because GTs are often difficult to overexpress in heterologous systems and the substrates of these enzymes, sugar nucleotides, are rather expensive to synthesize, making the chances of developing larger scale, economically-viable processes rather difficult to envisage. On the other hand, glycoscientists are increasing turning their attention towards enzymes, and in particular GHs, because these enzymes display a large spectrum of specificities, are widespread and are often readily available in recombinant protein form. One major drawback when using GHs for glycosynthetic purposes is that the mechanism of these enzymes allows for two reaction outcomes, which are hydrolysis and transglycosylation, with hydrolysis usually being the dominant outcome. Moreover, even when transglycosylation in GHs is sufficient to obtain measurable yields of transglycosylation products, the persistence of hydrolysis can lead to a secondary reaction (hydrolysis), which often radically diminishes the overall final transglycosylation product yield. Therefore, the challenge of engineering GHs for glycosynthetic purposes is diminish or eliminate hydrolysis, which in synthetic strategies is highly undesirable side-effect of the GHs catalytic mechanism, while maintaining or enhancing transglycosylation. Thereby, the acquisition of sufficient knowledge to allow the creation of non-Leloir furanose-transferring enzymes from an α-L-arabinofuranosidase.

In the absence of clear rules for rational design, we have used random and semi-rational techniques to tackle the engineering of a hydrolytic retaining GH toward TGs and we have recently succeeded using a combination of protein engineering strategies (i.e. random, in silico-driven, and site-saturation mutagenesis coupled with a reliable screening method) in creating a finely-tuned engineered GH that can be qualified as the first non-Leloir transarabinofuranosylases. When acting on simple nitrophenyl-activated donor sugars this enzyme displays an almost exclusive transglycosylating phenotype and transfers the sugar moiety onto acceptors at high yield (up to 80%). Moreover, the transglycosylation


products remain stable, notably thanks to a pH-control mechanism.\textsuperscript{3} Our achievements and recent analysis of accumulated bibliographic data,\textsuperscript{4} now provide us with a much clearer understanding of how the \textit{transglycosylation/hydrolysis} (T/H) partition could be established in GHs.

2. Aims of the PhD study program

The work that will be performed in this project will in many ways acquire further fundamental knowledge on carbohydrate-active enzymes and will thus extend our understanding of these enzymes and their activities. In particular the project will address questions linked to how some GHs are able to overcome thermodynamically-favoured hydrolysis when operating in aqueous media. If successful, the project will constitute a major step forward in the field, because despite many years of investigation the scientific community has not yet managed to define any generic rules to explain how GHs can effectively operate as TGs. Indeed, despite the very high similarity between family-related GHs and TGs it remains unclear how a GH can be converted into a TG, even though this fundamental question has been studied for at least twenty years and several hypotheses have been proposed. Therefore in order to increase the number of available TGs using protein engineering to transform GHs, it is necessary to better identify the molecular determinants that define the partition between transglycosylation and hydrolysis, and move towards the establishment of generic rules for future engineering of these enzymes. These are key challenges that will lead to game-changing technologies that will revolutionize the field of enzyme-mediated glycosynthesis.

In this research project, the aims are:

\textit{(i)} to acquire this understanding based on the molecular pathway designed to drive our well-studied enzymatic paradigm from a GH toward TGs and using ‘static’ crystallographic and ‘dynamic’ NMR approaches. The study of the enzymes/substrates interactions targeted separately different parts of the active site; notably based on a partially blocked active site, will also be developed using STD-NMR and ITC combined with ad hoc substrates.

\textit{(ii)} to apply the knowledge in order to create two new specific transglycosidase activities for syntheses that have so far mainly escaped the attention of mainstream research in this area (i.e. for the design of oligosaccharidic motifs containing rare sugars such as D-galactofuranose and D-rhamnose).

3. Research program

The project will be divided into two tasks: \textit{(i) generating new knowledge} concerning the route to convert our enzymatic model, an arabinofuranosidase from \textit{Thermobacillus xylanilyticus} (TxAbf), into transarabinofuranosylase and \textit{(ii) creating generic rules to engineer transglycosylation into glycoside hydrolases} based on the modification of substrate specificity of our paradigm enzyme and to the design of new TG from another GH.

\textit{- (i) TxAbf-based transglycosylases as enzymatic paradigm} (M0-M24).

This knowledge-generating task is intended to procure \textit{new insight into the molecular determinants that govern the T/H partition}, thus providing the basis for a generic strategy that will allow the creation of TGs that display both high transfer rates and yields, coupled to high product stability due to the absence of secondary hydrolysis. Relying on detailed \textit{structure-function relationships} at the amino acid level and knowledge about enzyme dynamics, this task will examine a set of mutant enzymes that have resulted from artificial evolution to probe the underlying effects that confer their properties and describe the incremental improvements that characterize them. This fine study of the evolutionary process will reveal clear amino acid level structure-function relationships and thus molecular level diagnostics of how transglycosylation has been progressively reinforced and


hydrolysis diminished. Ultimately, it is expected that the integration of the results obtained in this task will furnish a generic model that will describe how to convert GHs into TGs.

Mono-mutants of two selected amino acid positions will be created and expressed in *E. coli* and purified by standard methods. Afterwards, the transfer abilities (i.e. transglycosylation) of the different mutants will be assessed (including regioselectivity) using ¹H NMR. Detailed biochemical characterization for the evaluation of new site-specific mutations will be performed. Additionally, biophysical (STD-NMR and ITC) characterizations will be employed to provide a complete molecular level view of interactions and the consequences of previously studied and newly highlighted mutations. The different *TxAbf* mutants that represent the different milestones in the enzyme engineering trajectories (i.e. the final transarabino-furanosilases and the mutant intermediates leading up to them) will be studied using X-ray crystallography, working with apo-enzymes and complexed (with molecules that trap the covalent glycosyl-enzyme intermediate). Moreover, analysis by NMR of ¹⁵N-labeled enzymes will provide a complementary view of enzyme dynamics and especially modifications thereof in mutant enzymes.

- (ii) Generic ‘rules’ for enzymatic transglycosylation within GHs (M9-M32).

In this task, we propose to extend the range of artificial transglycosylating enzymes based on the evolutionary pathways that leads the *TxAbf* from hydrolysis to transglycosylation by modifying its substrate specificities toward the transfer of D-galactofuranosyl units. Furthermore, a similar approach, as a probable general route to engineer transglycosylation into another GH, either mannosidase or mannanase, will be applied. Highly conserved amino acids located around the active site will be carefully targeted.²⁵ The transfer abilities of these mutants will be monitored and the whole biochemical and biophysical characterizations will be performed for a clear understanding of the T/H balance within this new enzymatic case. It could be considered to use again a random mutagenesis approach and the screening strategy that is adapted from the approach developed by Koné et al. (i.e. sequential digital imaging-based evaluation of the *in vivo* hydrolise, *in vitro* acceptor activation and NMR-monitored T/H ratio).⁶

**Techniques to be learnt:** molecular biology methods (including PCR), protein biochemistry, enzymology (enzyme kinetics and NMR), biophysics (including X-ray crystallography, NMR of proteins, STD-NMR and ITC), glycochemistry.

- First (M12) and second (M24) thesis committee meetings, preparation of thesis manuscript (M30-M36) and thesis defense (M36). Twice, the student will present his bibliographic and experimental results to an external committee. The committee will provide an appraisal of the student’s work, which will be communicated to the CSC and the doctoral school as part of the yearly report. The minimum publication target is two publications before the defense with one or two publication likely to follow within 18 months of the defense.

4. Information on the LISBP housed in INSA Toulouse and supervisors’ team

The LISBP (*Systems Biology and Bioprocess Engineering*) is France’s top industrial biotechnology laboratory, being rated A+ in the last research evaluation exercise in 2014. It is a large laboratory that boasts nearly 300 staff, including 50-60 PhD students.

LISBP is sponsored by two of France’s major research agencies, CNRS (France’s biggest scientific research agency) and INRA (the second biggest agricultural research agency in the world), who have recently created a public-private partnership structure, Toulouse White Biotechnology, which works hand-in-hand with the LISBP to produce high level biotechnology-orientated research.

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The candidate will joined the group of Dr. Michael J. O’Donohue, one component of the Catalysis and Molecular Enzymatic Engineering’s team (CIMEs) led by Prof. Magali Remaud-Siméon. The activities of this group, which is currently staffed by 12 people, including 5 permanent scientists, is have focused both on the development of optimized hydrolytic enzymes for industrial applications, such as biorefining, and the investigation of transglycosytion in hemicellulases, with a view to developing new tools for glycochemistry. Additionally, the O’Donohue group has experience in enzyme engineering, notably using enzyme library creation and high throughput methods. The CIMEs’ team is extremely well equipped with state of the art equipment for the study and evolution of enzymes and, in particular, possesses a high throughput core facility (ICEO platform, Engineering and Screening for Original Enzymes, http://iceo.genotoul.fr) that is composed of liquid handling and colony picking robotic stations.

More information about the LISBP can be found by visiting this link: http://www.lisbp.fr/fr/index.html

Co-supervision of the PhD student will be performed by Dr. Michael J, O'Donohue, which possesses a BSc (hons) in Biomolecular Science (1987) and a PhD in Biochemistry, University of Portsmouth, UK (1991), as well as the Habilitation à Diriger des Recherches (University of Reims, FR, 2000). He is a specialist of protein structure-function studies and has worked on hemicellulases for 15 years.