N-Glycosyltransferase (ApNGT) and the *in-vitro* Glycosylation of γ-Sarcoglycan

Eston W. Blair

Oklahoma State University

The Honors College

Biochemistry & Molecular Biology

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A. Abstract

N-Glycosyltransferase of *Actinobacillus pleuropneumoniae* (ApNGT) is an enzyme which performs *N*-linked glycosylation of proteins. Furthermore, ApNGT is a cytoplasmic glycosyltransferase with the ability to target heterologous proteins. This is important as it can establish precedent for promising glycoprotein biotechnology *in-vitro* (1). At 631 residues in length ApNGT mimics a triangular topology. It has two terminals and three domains, and its binding pocket lies between an AAD domain and two Rossmann-fold domains (2). ApNGT's function is part of a growing body of protein glycosylation research (3). Residues His-277 and Gln-469 in the binding pocket are crucial for catalytic activity. It stabilizes the UDP-glucose and acceptor peptide in place and greatly influences the glycosylation site of the peptide (4). It is an effective post-translational modification enzyme with an ability to glycosylate complex proteins while also having relaxed sugar substrate specificity (5) (Reviewed in Ref 6). Yet, it greatly prefers UDP-glucose in the cytoplasm, uses an inverting mechanism, and recognizes the N*X*(S/T) sequence of the acceptor peptide or protein, where *X* is any amino acid but not proline (7). ApNGT also has a relaxed-substrate specificity and so is useful for a variety of acceptor peptide substrates, for example, human erythropoietin (1). We propose in this particular review NGT's ability to *in-vitro* glycosylate the membrane protein γ-Sarcoglycan. ApNGT allows for exploration of the structure and dynamics of glycosylated proteins. An

appropriate protocol includes the combination of ApNGT, UDP-glucose, and γ-Sarcoglycan to glycosylate the membrane protein. We can then perform NMR to identify structural, functional, and dynamic changes in the protein (8). This study will increase our understanding of how glycosylation affects the structure and dynamics of glycosylated proteins. It can also help with the diagnostics of various glycoprotein abnormalities.

B. Introduction

Glycosylation is a highly conserved process and has close involvement with protein function (2). Given its broad importance, it is critical to further our understanding of glycosylation: how it alters the function of a myriad of proteins (1). Studies in the recent past have approached glycosylation with experiments consisting of the basic reaction requirements. These requirements involve a glycosyltransferase, an acceptor peptide/substrate, and a hexose sugar such as UDP-glucose (6). Indeed, more complex synthesis of proteins have been demonstrated, combining various kinds of glycosyltransferases to extend a sugar scaffold on a protein, for example, polypeptide C34, a potent HIV inhibitor (5). However, there is little knowledge concerning how glycosylation affects membrane protein structure and dynamics, and further less how it alters the membrane proteins' function (7). Even when larger proteins are studied *in-vitro*, it is hard to determine precise measurements from NMR spectra (8).

In answer to this problem, we have developed a reliable experiment which reduces heterogeneity and can provide a more resolved NMR spectrum of a glycosylated membrane protein, γ-Sarcoglycan. While this methodology will be reported in a later study, our aim is to discuss the characteristics and role of ApNGT (7). The importance of this methodology translates into a novel understanding of membrane proteins, many whose altered function leads to degenerative or cancerous diseases.

C. ApNGT Characteristics

1. *Gene localization and organization*

The amino acid sequence for NGT in *Actinobacillus pleuropneumoniae* (ApNGT) is 631 residues long. It shares similar gene localization of HMW1C from *Haemophilus influenza*, the ortholog of ApHMW1C*. The gene *hmw1C* encodes for the glycosyltransferase (HMW1C) which performs HMW1 adhesin glycosylation. The gene *hmw1C* is accessory to *hmw1A*, the gene for the assembly of the HMW1 adhesin (2).

Recent studies indicate the high amino acid sequence similarity (85%) of the two proteins HMW1C and ApHMW1C. Though similar in their primary structures, *hmw1C* is clustered around HMW1C adhesin assembly and secretion genes. This cluster of genes creates a two-partner secretion system which synthesizes and protects the HMW1 adhesin. HMW1C glycosylates several residues of the HMW1 adhesin to protect it from host degradation (2).

Similarly, the gene organization of ApHMW1C† suggests its involvement in n-glycosyltransferase activity with post-translational modification of asparagine sites of HMW1 (10). The gene for ApHMW1C is positioned near open-reading frames of other glycosyltransferases, like glycosyl transferase, ribosomal protein RimO, and a nucleosidase (9).

*synonymous with ApNGT

†GenBank: ABN74719.1 (2010, The *Actinobacillus*)

Thus, ApHMW1C, now known as ApNGT, is distinct from its ortholog by its position in the bacterial genome, as HMW1C is positioned in a locus constituting a two-partner secretion (TPS) system (10).

2. *Protein Structure*

The structure of ApNGT contains three domains. The structure was first determined in 2011, showing it was a medium sized protein (~70kD) containing an N and C terminus, a catalytic site, and a narrow gap for the acceptor protein (2)(10). It has a triangular face, viewed from the side, formed by the AAD fold at the "top" and GT-A and GT-B folds on the "bottom." Its three-dimensional crystal structure can be found with the PDB ID of 3Q3H (2) (Fig. 1). The protein, as all proteins are, is divided into two termini with secondary structures. The N-terminal contains an all-alpha domain (AAD) of thirteen repeating alpha helices. Together, the AAD forms the "head" of the enzyme, above the catalytic site in relation to the C-terminal end. The C-terminal contains a GT-B fold, encompassing two domains, named GT-1 and GT-2, which have secondary structures similar to Rossmann-folds, alternation of alpha-helices and beta-sheets (2) (Fig. 2). Together the GT-1 and GT-2 folds are similar in secondary structure and form the "bottom" of the enzyme. Nestled within the GT-2 domain is the binding site. Together, ApNGT is a glycosyltransferase whose GT-B domains characterizes its catalytic activity.

The GT-B fold is metal-ion independent and characteristic of the GT-41 family (10). The GT-B fold differs from the GT-A fold in two ways. The GT-A fold glycosyltransferases use metal-ion dependent catalysis (Manganese is typically observed) and have a conserved DXD* motif, which helps with sugar binding in their binding site. In contrast, GT-B folds are metal-ion independent and do not have a conserved DXD motif. GT-A folds have one similar domain which contains Rossmann-folds, in contrast to the GT-B fold which has two similar domains. Yet, both GT-A and GT-B glycosyltransferases contain Rossmann-folds and sugar binding sites near these Rossmann-folds (Reviewed in Ref 11). These variations in the folds constitute the diversity of glycosyltransferases, and this further explains the nature of the binding site.

The binding site is characterized by a narrow hole near the center of ApNGT. The interface between the GT-2 and AAD domains create a narrow groove adjacent to the binding site, whose length end is 7 angstroms and wide end is 18 angstroms. This is just large enough for proper alignment of UDP-hexose in the binding site. Conserved hydrophobic residues of both the AAD and GT-2 domain line this narrow groove, essential for retaining the acceptor peptide or protein and UDP-hexose. The proximity of the AAD and GT-B domain suggests its originality and influence on the catalytic site (2).

In ApNGT, the singular catalytic site resides in an interdomain between the GT-1 and GT-2 domain, spatially more interactive with the GT-2

domain. During the binding of a UDP-hexose, two configurations are possible. These configurations induce small, local conformational changes (hydrophobic and Van der Waals) around the binding site to allow appropriate binding of the UDP-hexose. Site-directed mutagenesis supported the importance of certain binding site residues and the narrow groove for catalytic activity (2). There was almost no catalytic activity when the well conserved Lys-441, Asn-521, and Asp-525 were deleted (2)(6)(Fig. 3 – 5). These amino acids affect binding the most, because they interact with the ribose and phosphate moieties of the UDP-hexose (12) (13). Lys-441, Asn-521, and Asp-525 interact with the two phosphate groups of UDP-hexose, stabilizing it through hydrogen-bonding and resolving hydrostatic clashes (2). Though the catalytic site is where glycosylation occurs, equally important is the site which holds the acceptor protein during catalysis. The site which accepts the UDP-hexose and acceptor protein is the AAD fold and the GT-1 domain of the GT-B fold. Thus, the catalytic site of ApNGT is suited in such a way for appropriate binding of the hexose moiety of UDP-hexose to the asparagine of the acceptor peptide or protein.

3. *Post Translational Modifications*

ApNGT is a post-translational modification enzyme which performs N-linked glycosylation. N-linked glycosylation is the attachment of a sugar moiety onto an asparagine residue of an acceptor protein (Reviewed in Ref 3)(12)(13). The consensus sequence for ApNGT is NX(S/T) (on the acceptor protein), where *X* is any amino acid but proline (7). The attachment takes place through a bond between the nitrogen atom of the asparagine amide group and the anomeric carbon of the hexose sugar. Glycosyltransferases assemble hexose sugars onto proteins to form glycans, a complex of both sugar and protein. These hexose sugars may undergo sequential elongation by other glycosyltransferases such as alpha-6-glucosyltransferase (4)(7). With elongated sugar scaffolds, future studies can address how this affects the structure and function of proteins. This constitutes a promising exploration for glycoengineering. Glycosyltransferases are found in all three domains of life, but N-linked glycosylation is most prevalent in eukaryotes (14) (15). Half of all known eukaryotic proteins are modified by N-linked glycosylation (16). Glycosylation of proteins is involved in cell to cell signaling, signal transduction, protein folding, and interactions with viruses, to name a few (17)(18)(19). Thus, the study of ApNGT provides a way into the study of glycan structure and dynamics and how it might play a role in these functions.

4. *Protein Function and Activity*

   a. Background

ApNGT catalyzes the transfer of a hexose-sugar from a nucleoside-sugar toward an asparagine site in an acceptor protein to form a protein (7). The discovery of ApNGT's subfamily (of GT41) activity came through mass spectrometry of glycosylated HMW1 adhesins. HMW1C was found to attach, through *N*-linked glycosylation, hexose sugars onto thirty-one asparagine sites in the HMW1 adhesin. The HMW1 adhesin is a pathogenic factor whose glycosylated surface protects it from premature degradation. The attachment of sugars onto glycans was novel at the time, as most proteins were thought to have been modified through *N*-acetylation. Determining the structure for HMW1C has been difficult through NMR, because of its size. Thus, a homolog of HMW1C was found in *Actinobacillus pleuropneumoniae* (20). This enzyme differs from known glycosyltransferase activity in the periplasm of prokaryotes called O-OST modified glycosylation (21) (3). Biomolecular fluorescence complementation studies determined that NGT's form both homo and heterodimers in live cells, localize in the Golgi apparatus, and attach *N*-glycans through post-translational modification (22). In addition, a lipid core is not needed as part of the hexose substrate, as seen in most bacterial *N*-glycan and LPS biosynthesis (21) (3). Bacterial *N*-glycan

biosynthesis and glycan biosynthesis in the endoplasmic reticulum is performed through *en bloc* glycosylation, which uses a lipid-linked oligosaccharide (3). ApNGT, in contrast, uses sequential glycosylation of sugars onto proteins in the cytoplasm (1)(3). This uncomplicated homolog of HMW1C, then, can be applied to glycosylation studies, having less requirements for the enzyme (1)(4).

b. Substrates

The homolog of HMW1C, known as ApNGT, glycosylates proteins with the hexose sugar substrates of glucose and galactose to Asn sites in acceptor proteins (10). ApNGT highly favors UDP-glucose as a substrate donor (Fig. 6) (10) (6). This is important because UDP-glucose is fairly inexpensive. In addition, it prefers monosaccharides like UDP-galactose, UDP-xylose, and GDP-glucose (surprisingly) but not polysaccharides such as UDP-mannose (Fig. 7) (21) (6) (5). The advantage of using monosaccharides is noise reduction in NMR spectra when determining the structural changes as a result of glycosylation. It also has relaxed specificity for substrate peptides and can glycosylate heterologous proteins (1). This is important because these types of proteins are highly prevalent in pathways pertinent to membrane dynamic studies and abnormalities such as cancers and

diseases. The relaxed peptide sequence specificity and preference of UDP-glucose characterizes ApNGT's utility for *in-vitro* assays.

c. Mechanism and Kinetics

During attachment of the sugar onto the acceptor protein or peptide, ApNGT uses an inverting mechanism and recognizes the N*X*(S/T) sequence of the acceptor protein. This sequence provides a reliable sequon for targeting proteins. ApNGT catalyzes the beta-configuration of the bond between carbon one of glucose and the amide nitrogen of asparagine, as determined by NMR (7)(Figure 8). ApNGT highly prefers UDP-Glucose (5). The N*X*(S/T) sequon has the highest rate of turnover with UDP-Glucose as the substrate (k_{cat}/K_m = 502.8 $M^{-1}s^{-1}$), and prefers threonine over serine of the acceptor sequence by a factor of ten (6). Basic residues which surround the pocket do not affect the kinetics, for when they were mutated to alanine there was no change in catalytic activity (6). ApNGT can be inhibited by epoxy-peptide derivatives such as 2,3-epoxypropylglycine and epoxyethylglycine (23). These derivatives induce the enzyme to catalyze its own inactivation only when glycosylation occurs. Overall, ApNGT has the favorable trait of a fast turnover of UDP-glucose for n-glycosylation.

d. Application

ApNGT exhibits these characteristics stated above in the cytoplasm of *E. coli* (1). This is important for vaccine or diagnostic purposes, as its functional transfer into *E. coli* makes available a reliable experimental system (1). However, ApNGT has a relaxed substrate specificity, which is problematic in studying specific sites on glycosylated proteins. Yet, In addition, ApNGT cannot build complex sugar scaffolds, limited to glycosylation of one asparagine residue (6)(7). Yet these limitations are not significant overall, as it serves as an efficient enzyme for rapid glycosylation of desired proteins *in-vitro* (5)(7). This has already been demonstrated on various proteins and synthetic peptides (1). Some examples of modified proteins include: virus envelope glycoprotein, ribosome inactivating protein, hemagglutinin, and hydrolase inhibitor (24). In addition, both ApNGT's cytoplasmic glycosylation role and role in virulence can be applied to glycoengineering, as stated previously (25). ApNGT glycosylated proteins can serve as primer sites for sugar scaffolding (Fig. 8) (7). ApNGT has successfully glycosylated several proteins of interest: Glyceraldehyde-3-phosphate dehydrogenase, Phosphoenolpyruvate carboxykinase, Cholera toxin subunit B (CtxB), human erythropoietin (1). Thus, ApNGT can be used with a variety of proteins and the full extent of its application is still unknown.

5. *Relationship between Structure and Function*

The relationship between ApNGT's structure and function is determined mainly by its binding core. As stated before, the turnover rate of ApNGT is affected by two crucial residues which affect substrate specificity: His-277 and Gln-469 (4). A mutant of ApNGT (Q469A), when Val, Ala, Ser, Leu, Phe, Gly were substituted at the Gln-469 site, had a higher turnover rate when assayed with the optimal peptide acceptor sequence ANVTK. Gln-469 highly influences UDP-hexose, holding its position in the active site (4). His-272, His 277, Arg-281, Lys-441, and His-371 are basic amino acids located near but not necessarily needed for catalysis within the active site (6). The surface of the enzyme is electrostatically negative, with most hydrophobic residues forming alpha helices. The binding pocket is more electrostatically positive, as expected with basic residues such as histidine, lysine, and arginine. His-277 stabilizes the secondary phosphate of the UDP-glucose, such that C_1 of the glucose faces outward from the groove to where an Asparagine of the peptide may bind. Among other residues in the binding pocket, His-277, His-272, Arg-281, His-371, His-495, and Lys-441 all are highly conserved among other OGT, including *H. sapiens* OGT (6). Interestingly, though ApNGT has a separate mechanism than that of most OGT's, both share the same substrate specificity (6). Perhaps the conserved, basic residues within the binding pocket indicate a conserved presence of stabilizing the UDP-glucose. It is still unclear how the mechanism of ApNGT

may occur, although His-277 and Gln-469 play a major role as said previously. In addition, the quaternary structure features surrounding the binding pocket allow for the proper positioning of the peptide, by hydrophobic interactions of alpha helices.

D. ApNGT and the *in-vitro* glycosylation of γ-Sarcoglycan

1. *Description*

In-vitro glycosylation of proteins involves an acceptor peptide or protein, a sugar substrate, and a glycosyltransferase. Mass spectrometry and NMR studies can indicate the changes in dynamics and structure of the protein as a result of the attachment of these glycans (8). Its application is wide, as most proteins are glycosylated in post-translational modification. While recent studies have shown successful glycosylation of peptides *in-vitro*, none have been successful using membrane proteins. Few proteins have been glycosylated, and less still is our understanding of how glycosylation affects protein structure and activity. Structural determination of glycosylated proteins is hindered often by poor expression, diffraction, glycosylation, heterogeneity of the sample, and severe chemical shift overlap (8). Further still, no successful attempts have been made to glycosylate a membrane protein *in-vitro*, which present their own set of problems.

2. *Proposal of the in-vitro Glycosylation of γ-Sarcoglycan with ApNGT*

Our group has successfully glycosylated a membrane protein, γ-Sarcoglycan, *in-vitro*, with research to be published soon. Through mass spectrometry we were able to detect the mass shift differentials which demonstrates we have successfully glycosylated the protein. In addition, we are working to determine the structure of γ-Sarcoglycan through NMR

methods as used previously (8). We overcame specific difficulties of glycosylation related to the use of lipids and detergents, which could have affected the glycosylation of γ-Sarcoglycan. Specifically, NGT was used because of the characteristics described previously. It was used mainly because of its size, preference of UDP-Glucose *in-vitro*, and selective preference for the acceptor sequon NX(S/T) (6). This process can be applied to many membrane proteins, opening up more possibilities in glycoengineering, for vaccination and disease detection methods (3)(1)(4)(5)(24)(25). Glycosylating γ-Sarcoglycan may also serve as a model with which to further understand Duchene Muscular Dystrophy.

E. Conclusion

In total, ApNGT has a high potential in glycoengineering, favorable to a variety of further studies of human and prokaryotic proteins. With its relaxed substrate specificity, its strong preference for UDP-glucose, and relatively small size, it is an effective and versatile enzyme (1) (7). Further studies may involve determining: its catalytic mechanism, what conditions suppress or induce gene expression, and interaction with other glycosylation enzymes (1) (3) (4) (24) (25). Most importantly, ApNGT can be used effectively in novel *in-vitro* glycosylation studies involving a myriad of proteins.

F. Figures

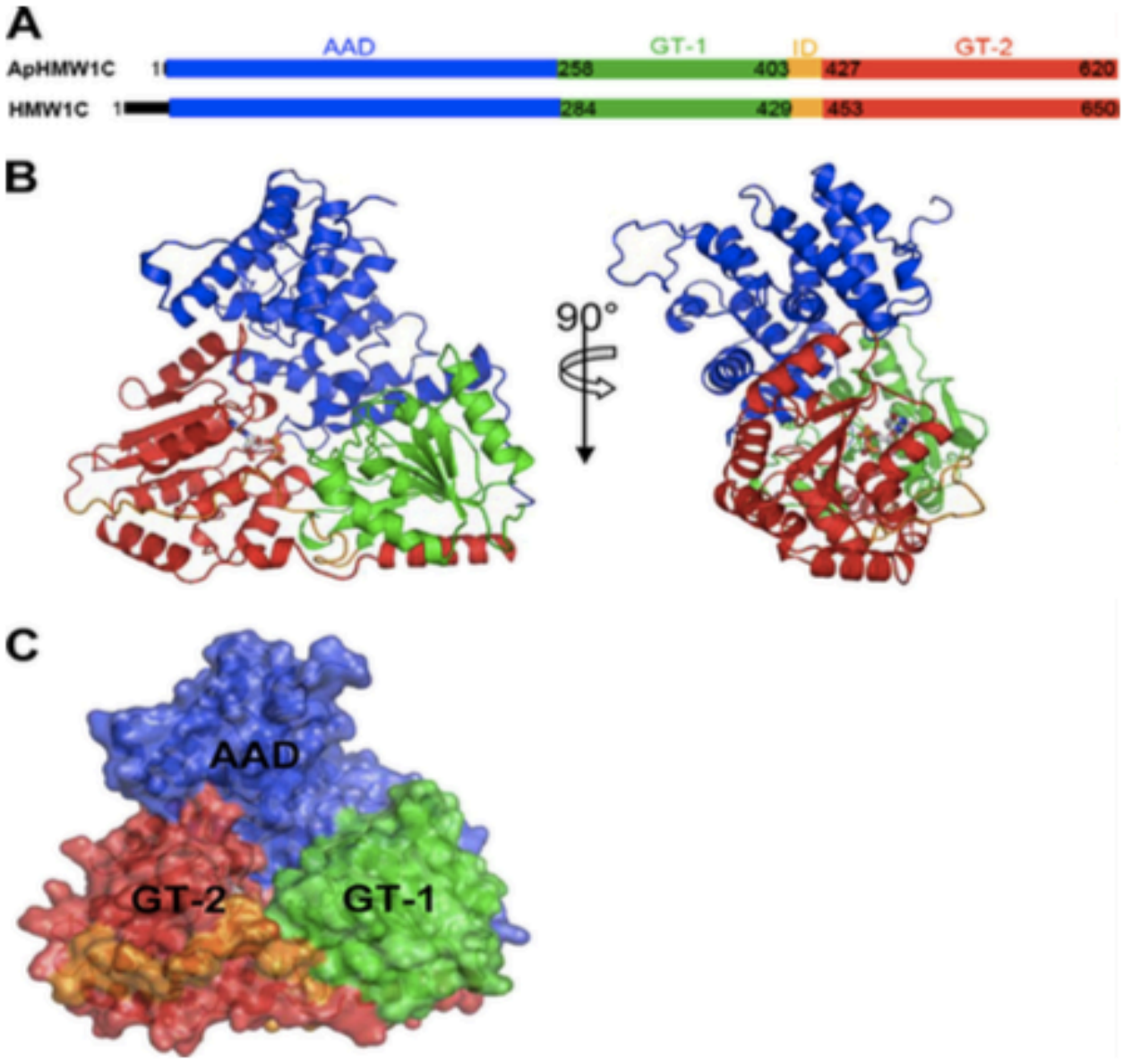

FIGURE 1 – (Reference number 2)

A. A domain comparison map of ApHWM1C (ApNGT) and HMW1C
B. A ribbon model illustrating the structure of ApHWM1C (ApNGT)
C. A space fill model of ApNGT divided into AAD, GT-2, and GT-1

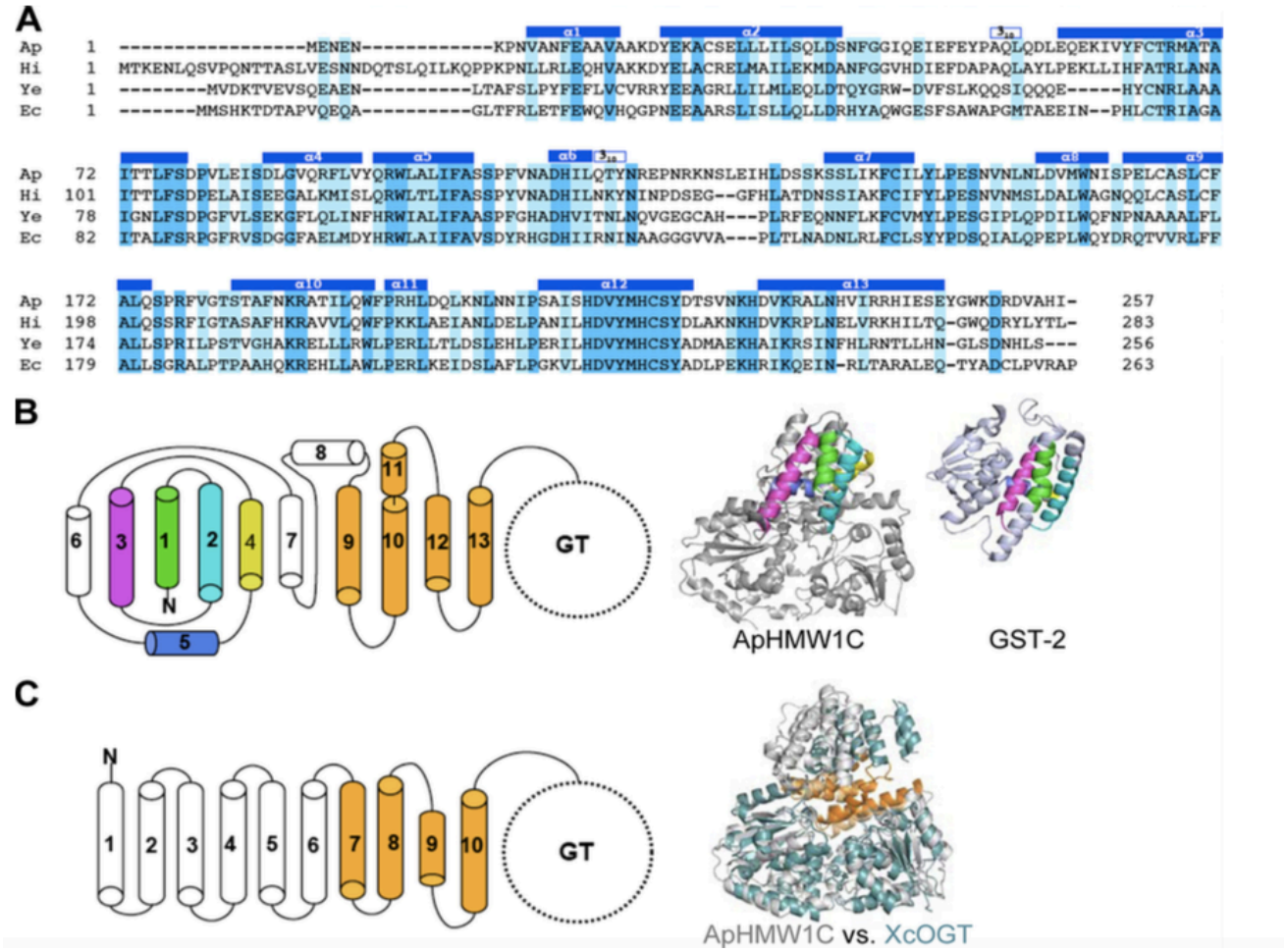

FIGURE 2 – (Reference number 2)

A. An alignment of the N-terminal domain AAD showing conservation among *A. pleuropneumoniae* (Ap), *H. influenzae* (Hi), *Yersinia enterocolitica* RcsC (Ye) and *Escherichia coli* EtpC. Identical alignment is shown in blue and conserved is light blue
B. A topology map of the N-terminal domain - alpha helices are shown as cylinders, and the ribbon models illustrate the position of the alpha helical domain on ApHMW1C (ApNGT) and GST-2
C. A topology map of the N-terminal domain and TPRs (gold) of XcOGT, and a superimposition of ApHMW1C (ApNGT) and XcOGT models. Note the difference between the N-terminal domains (AAD), shown in light gray, and the similarity of the TPRs, shown in gold

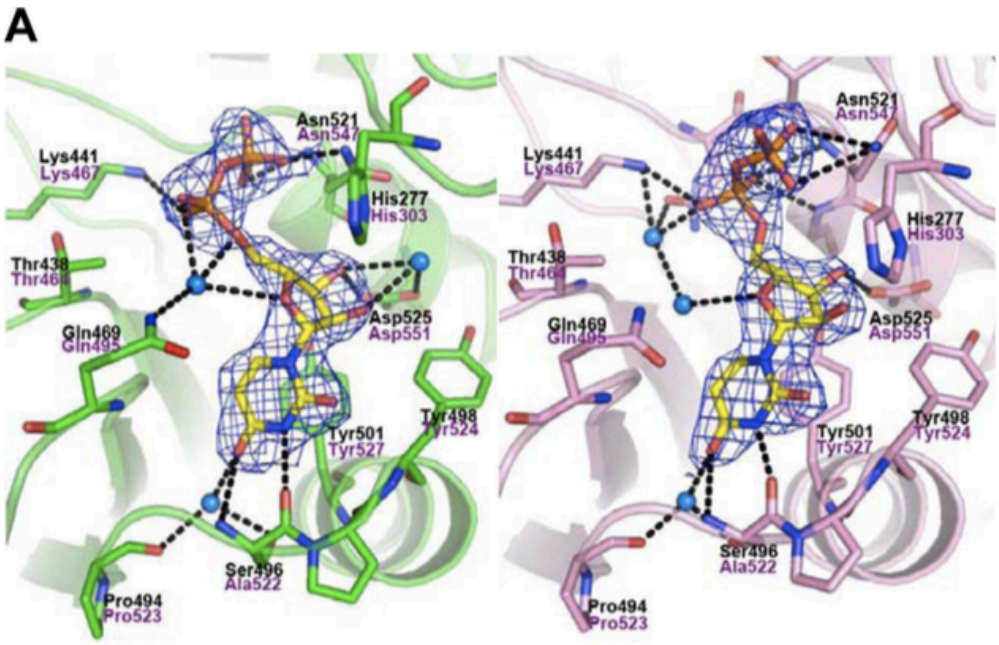

FIGURE 3 – (Reference number 2)

A. Two conformations of UDP-glucose from two protomers are shown in the binding pocket, with essential residues (stick) indicated in bold black (ApHMW1C) and magenta (HMW1C). Water molecules in blue are shown, with black dashed lines indicating hydrogen bonding.

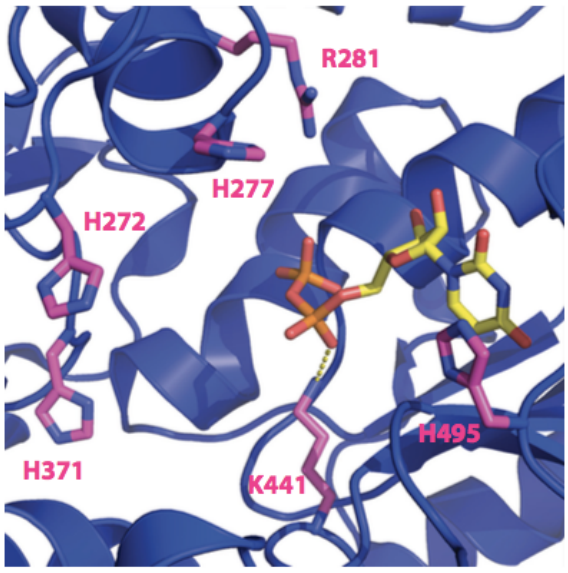

FIGURE 4 – (Reference number 6)

A crystal structure of ApNGT and UDP, with key residues highlighted in magenta, indicating a basic interaction of less than 8 angstroms from the ß-phosphate of UDP.

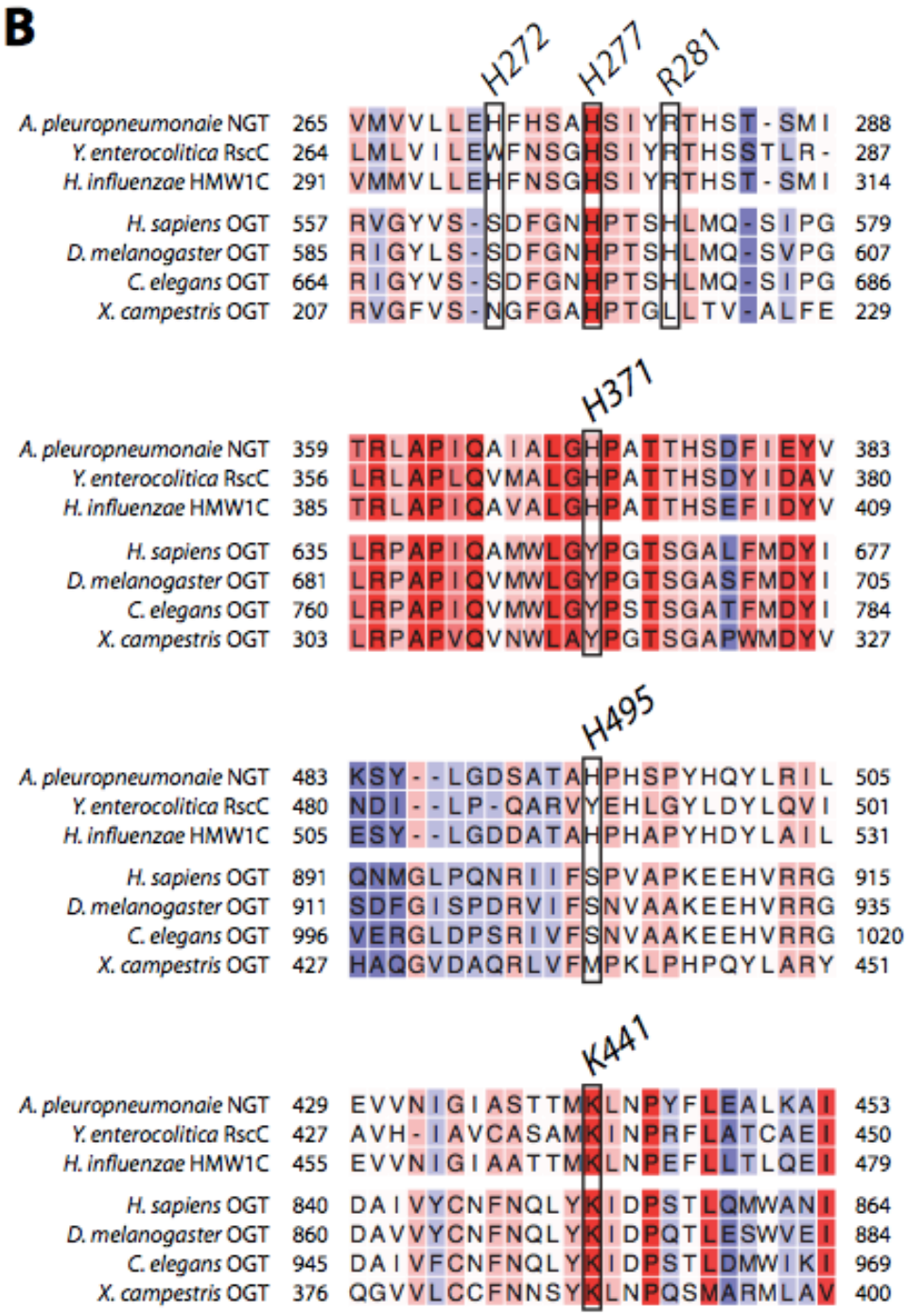

FIGURE 5 – (Reference number 6)

A genetic sequence map showing the conservation of catalytic residues His-272, His-277, Arg-281, His-371, His-495, and K-441 among various glycosyltransferases.

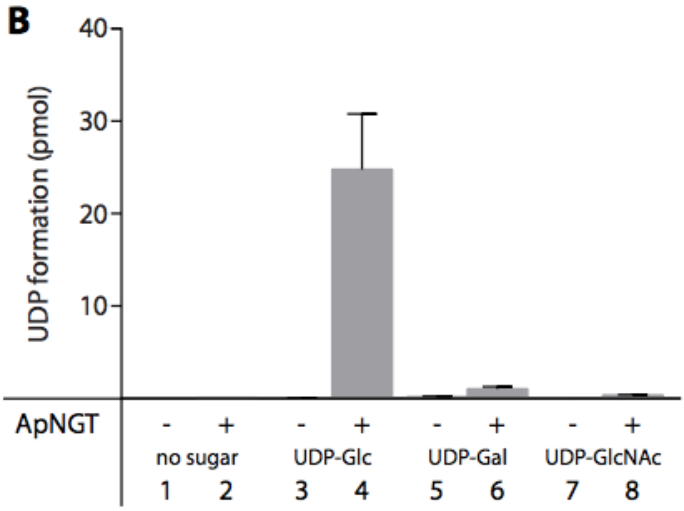

FIGURE 6 – (Reference number 6)

A graph showing the amount of UDP formation from the reaction of ApNGT and sugar substrates UDP-Glc, UDP-Gal, and UDP-GlcNAc.

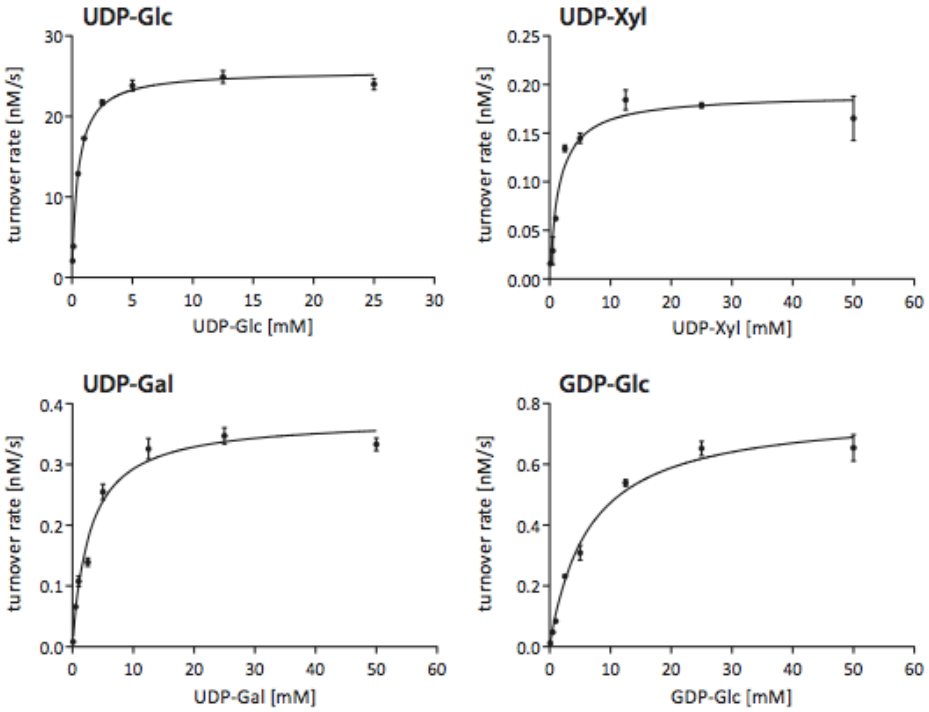

FIGURE 7 – (Reference number 6)

A graph illustrating the glycolytic turnover rates of UDP-Glc, UDP-Xyl, UDP-Gal, and GDP-Glc by ApNGT. Note the magnitude of the turnover rate values.

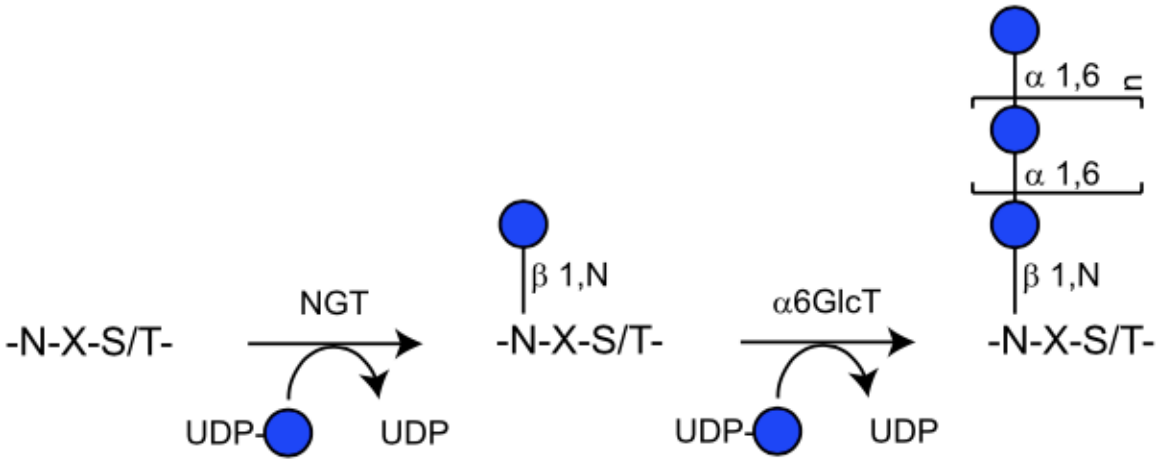

FIGURE 8 – (Reference number 7)

A picture showing the method by which glycan scaffolds are developed. The acceptor sequence N-X-S/T is the base upon which NGT and *α*6GlcT glycosylate UDP-hexose (blue circle) onto the Asn residue/previous hexoses.

G. Acknowledgements

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H. References

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