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Tailoring Enzymatic Reactions

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Abstract

Carbohydrates remain as one of the most challenging fields of study mostly due to the immense catalogue of carbohydrate structures that can be obtained as a direct result of their non-template dependent synthesis. Owing their chemical nature, there exist a huge number of potential combinations for glycostructures with different complexity which makes their synthesis difficult and almost impossible in most cases using only a traditional chemical synthesis approach.

As glycans are involved in distinct physiological roles including energy store, infection, pathogenesis and cell signalling among other processes, studies dealing with carbohydrates synthesis and regulation have increase within the last decades. Furthermore, carbohydrates as well as other glycosylated compounds including antioxidants, drugs and different proteins find application in the food and cosmetic industry as well as in health care, thus controlled glycosylation is targeted in order to develop new potential therapeutic agents as well as functional foods ingredients.

A strategy combining protein and substrates engineering as well as classical chemical synthesis has been necessary in order to achieve the synthesis of tailor-made oligosaccharides and glycostructures. Different approaches are described in this review regarding the control of chemo-, stereo- and regio-selectivity of carbohydrate active enzymes that successfully allowed the synthesis of an expanded spectrum of new glycostructures including those synthesized from non-natural substrates.

Introduction

Among the four major classes of organic molecules in living systems, carbohydrates are the most abundant molecules in nature [1]. Carbohydrates are the primary energy-storage molecules in living organisms and play a crucial role in biological processes and microorganism's survival. Sugar polymers are involved in bacterial adherence, protection of microbial cells against desiccation, metal ion stress, antibiotics, toxic compounds, phagocytosis and phage attack [2 – 4]. In eukaryotic cells, complex carbohydrates are found covering the major part of the cell surface and are integral part of the extracellular matrix of epithelial cells [5]. Glycans are involved in many physiological and pathological events, including cell growth, migration, differentiation, tumour invasion, host-pathogen interactions, cell trafficking, and transmembrane signalling [6]. The expression of complex carbohydrates in glycoproteins, glycosphingolipids, proteoglycans and glycosylphosphatidylinositol-linked proteins is one of the most common post-translational modifications [6, 7].

Carbohydrates are one of the most challenging fields of study mostly due to the immense repertoire of carbohydrate structures that can be derived from its non-template dependent synthesis. While the synthesis of bacterial homo-polysaccharides usually requires the action of a single enzyme, eukaryotic glycosylation is far more complex involving dozens of genes. Glycosylation of proteins and lipids is carried out by a combined and ordered sequential action of glycosyltransferases and glycosidases in eukaryotic cell compartments such as the rough endoplasmic reticulum and Golgi apparatus [7]. The complex heterogeneity of carbohydrates is a direct result of several of their intrinsic characteristics: the ability of different types and numbers of sugar residues to form glycosidic bonds with one another, the structural characteristics of these molecules, the type of anomeric linkage, the position and the absence or presence of branching [1]. Due to these characteristics there exist a huge number of potential combinations for glycostructures with different complexity and properties.

Carbohydrates as well as other glycosylated compounds including antioxidants find application in the food and cosmetic industry as well as in health care. Increasing attention has been focused on diverse carbohydrates due to their nutraceutical properties. On the one hand natural and unnatural-occurring sugars are common ingredients in the so called functional foods. The world market for functional foods has been continuously growing during the last three decades, with an estimated size in 2003 of about US$ 33 billion, while the estimation for the European market exceeded US$ 2 billion in the same year [8]. On the other hand glycosylation of diverse molecules is targeted in order to develop new potential therapeutic agents including vaccines, glycoprotein therapeutics and glycosylated drugs. Glycosylation of bioactive and pharmacologically active compounds represent a challenge in the synthesis and diversification of carbohydrate-active molecules.

The synthesis of oligosaccharides and polysaccharides becomes a challenging goal for modern science as the high complexity of these molecules makes their production difficult by classical chemical synthesis [9]. Nevertheless, enzymatic and chemo-enzymatic synthesis of glycans using carbohydrate active enzymes appears as the most attractive option to overcome these drawbacks including selectivity. Thus, the production of tailor-made oligosaccharides and polysaccharide structures relies on the elucidation of the enzyme structural determinants driving chemo-, stereo- and region-selectivity, as well as on solvent and substrate engineering.

Beside Leloir-type glycosyltransferases that catalyse the synthesis of different carbohydrates using nucleotide-activated sugars as substrates, bacterial glycosyltransferases which catalyse the synthesis of various large glucose polymers from sucrose provide an alternative route for the synthesis of targeted glycostructures [10]. To date success in the field of enzyme specificity control has been achieved by a combination of substrate and genetic engineering using enzymes such as levansucrases from *Bacillus subtilis*, *Bacillus megaterium* and a glucansucrases from *Streptococcus oralis* among others [9 – 15].

Tailor-made Oligosaccharides and Fructo-oligosaccharides

Within the last 20 years, studies dealing with nutraceutical and functional foods have rapidly increased and so have the sales of products containing them. Several papers have been published so far describing the effects of oligosaccharides and glycosides in human health and nutrition, focus the crucial role that the degree of polymerization plays in sugars characteristics and physiological effects [16 – 18].

To date oligosaccharides included in several commercial products are obtained by controlled hydrolysis of large polymers usually extracted from plants and requiring additional purification steps, or by microbial synthesis using either β-D-fructofuranosidases or fructosyltransferases mostly from *Aspergillus* species.

The search for new carbohydrate synthesizing enzymes that allows the synthesis of specific groups of oligosaccharides as well as a broader range of glycosides has lately intensified pointing towards bacterial fructosyltransferases. These enzymes belong to the family 68 of glycoside hydrolases (EC 2.4.1.10) and synthesize fructose polymers with different linkage specificity and polymerization degrees using sucrose as natural substrate. Fructosyl-transfering enzymes are divided in levansucrases (LS) which synthesize a β2,6 linked polymer known as levan and inulosucrases which mainly form inulin, a β2,1 bound product. Although crystallographic structures of three levansucrases and one inulosucrase are now available including structures with different substrates, to date there is not enough information highlighting the differences either in the active site or in the nearest sub-sites that may support or influence linkage specificity in fructosyltransferases [15, 19, 20].

However, a model to explain the polymerization mechanism in polymer and oligosaccharides producing fructosyltransferases suggests that polymer synthesis obeys to a processive transglycosylation reaction, while the synthesis of oligosaccharides results from a nonprocessive mechanism controlled by specific amino acids in the catalytic domain [21]. Nevertheless, additional sub-sites beyond -1 (fructose residue) and +1 (glucose residue) where sucrose binding takes place were not identified. In spite of having the same active site architecture, *B. subtilis* and *B. megaterium* (SacB) LSs mainly synthesize high molecular weight fructans, whereas the homologous enzyme from *G. diazotrophicus* only synthesizes oligosaccharides with a minor amount of oligosaccharides.

Homann *et al.* [13] clarified the functional roles of different amino acids which are involved in the formation of different reaction products including those controlling the polymer molecular weight and turned the polymer synthesizing SacB from *Bacillus megaterium* into an oligosaccharide-producing enzyme, thus changing from a processive to a non-processive mechanism. Multiple sequence alignments of LS enzymes in combination with structural data of family 68 members allowed selection of the residues Trp94, Asp95, Val115, Leu118, Trp172, Ser173, Asn252, Arg256, Asp257, Glu350, Glu352, Arg370 and Tyr421 to explore the structural determinants which are responsible for the transfructosylation activity in *B. megaterium* SacB. Among the studied residues Asn252 was found to play an important role in the transfructosylation reaction, as the variant Asn252Ala completely stopped the polysaccharide production by reducing the coordination of a fructosyl unit in the +2 sub-site and being only able to forms short-chain oligosaccharides of up to three fructosyl units (nystose). Derived from biochemical studies of the constructed mutants and product structures determination, it was proposed that a high affinity in the sub-sites +2 and +3 is needed for binding the growing fructan chain in polymer synthesizing enzymes. Asn252 is strictly conserved in Gram-positive bacteria, whereas in Gram-negative bacteria this region shows variability. Additionally, it was the first example of an engineered FT bearing different product specificity without significantly changing its K_m and k_{cat} values.

Later on Strube *et al.* [15] demonstrated that polysaccharide synthesis in SacB is controlled by surface motifs beyond the sucrose binding site. Variants Y247A, Y247W, N252A, D257A, and K373A were characterized and their structures obtained at resolution between 2.0 and 1.75 Å, finding distinguishable sub-sites for polysaccharide synthesis. As the active site was found to remain unaltered by single mutations, changes in the transfructosylation products were only attributed to the direct effect of each mutation.

N252A, K373A and Y247A variants were found to eliminate the polysaccharide production after tetrasaccharides, hexasaccharides and decasaccharides synthesis respectively. These three amino acids form a platform for a possible stabilization of the acceptor fructan chain. The biochemical and the structural data show that Asn252 is located close to the sucrose binding site, whereas Lys373 and Tyr247 are clearly apart from the sucrose binding cavity of SacB. A deeper view into the role of the residue Lys373 revealed that the exchange of lysine

in position 373 to arginine, leads to longer oligofructosides (9 fructosyl units). It was observed that although interactions between the functional amino groups of arginine in position 373 and the amino acid network as well as carbohydrate units are still possible, the interactions are reduced compared with the wild type enzyme. These changes stop the polymer formation. Additional mutants in the residue Y247 were also constructed, finding that SacB variants Y247A and Y247I form oligosaccharides consisting of up to nine fructosyl units, whereas variant Y247W forms the whole range of oligosaccharides compared with the wild type spectrum. These results indicate that an unpolar favoured stacking mechanism is possible with tyrosine as well as tryptophan in this location but not with alanine or isoleucine. Superposition of Y247W with D257A (the transition state stabilizing residue) confirms the key supporting role of Tyr247 in the oligo- and polysaccharide-forming "assembly line." As previously reported, *G. diazotrophicus* levansucrase LsdA [22] forms mainly the β-(2,1)-linked trisaccharide 1-kestose, whereas the wild type SacB forms mainly polysaccharides. A structural comparison of both levansucrases enabled further insights into the functional role of Tyr247. A conformational difference in the surface motif Tyr247 was observed to be essential for polysaccharide synthesis in SacB, while the orientation of this motif in LsdA might block the polysaccharide chain, thus leading to the synthesis of short oligosaccharides of 3 – 5 carbohydrate units.

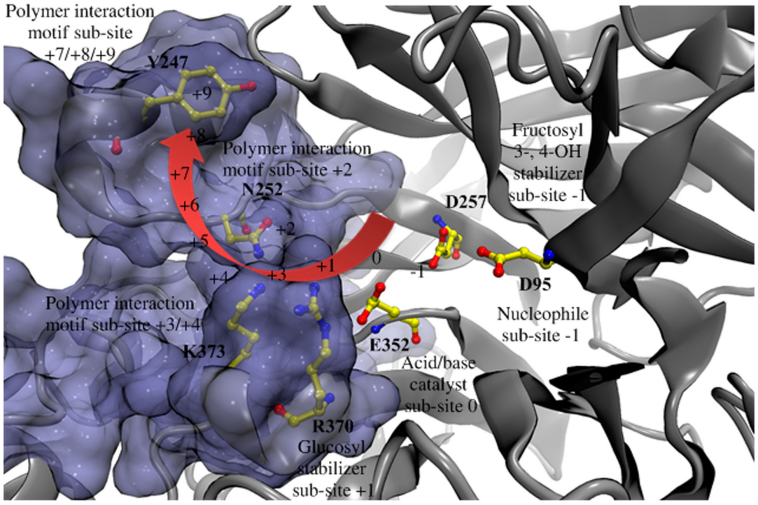

Figure 1. Potential pathway for the synthesis of oligosaccharides in *B. megaterium* SacB [13].

It was shown for the first time that amino acids outside the active site of a levansucrase have a well-defined and rationally explainable effect on the polymer formation activity (Figure 1). Due to the extensive structural data obtained from SacB variants Y247A, Y247W, N252A, and K373A any effect on the position of other amino acids was be excluded. Conformational

analyses of variants Y247A, Y247W, N252A, and K373A revealed an unaltered active site architecture that was consistent with the kinetic parameters of these variants which were not significantly different compared with wild type SacB.

Based in these studies, it may be concluded that the identification of the sub-sites +2 to +9 enables now the synthesis of tailor-made short-chain fructo-oligosaccharides and opens the gate to similar studies on diverse levan and inulosucrases in order to fulfil the needs for functional carbohydrates to different applications.

Changing Linkage Specificity

It has been extensively reported that both the glycosidic bond specificity and carbohydrate degree of polymerization are two characteristics that strongly influence the carbohydrate fermentation during its transit through the digestive tract. Thus, the physiological effects, which are derived from this carbohydrates selective fermentation, are directly related with both properties underlying the importance of modulating sugar stereo- and regio-selectivity. Sanz *et al.* [23] studied the influence of both parameters on selective fermentation of glucose oligosaccharides by human gut bacteria. Glucobioses with α and β1,2 linkages showed to be particularly selective for bifidobacteria, and in general, α-glucosyl-glucose disaccharides showed more positive effects than β-isomers. *trans*-Galactooligosaccharides with linkages β1,6 and β1,3 have been shown to be selective for bifidobacteria, the β-galactosidases of which cleave such isomers faster than β1,4.

Streptococcus oralis dextranssucrase engineering has recently proved to be efficient for tailoring enzyme regio-specificity [9]. Glycosyltransferase R (GTFR) from *S. oralis* is a dextransucrase belonging to glycoside hydrolase family 70. Besides glucan production, GTFR catalyses the transglycosylation of different acceptor substrates, including alcohols and amino acids. Depending on the specific enzyme bacterial glucansucrases synthesize a variety of glucosidic linkages in their products, forming either α1,6-linked dextran, α1,3-linked mutan, α1,4 linked reuteran or an α1,3 – 1,6-alternating alternan. Two questions were recurrent about glucansucrases: how they control either oligosaccharide or polysaccharide synthesis and how their glycosidic linkage specificity is directed [9]. This information is required for the production of tailor-made saccharides.

Kralj *et al.* suggested that [14] variations in glucosidic linkage specificity appear to be based on relatively small differences in amino acid sequences in their sugar-binding acceptor sub-sites. This assumption was derived from mutagenesis of some amino acids of *Lactobacillus reuteri* 121 GTFA (reuteransucrase) probably involved in acceptor substrate binding. A triple mutant N1134S, N1135E and S1136V in a region immediately next to the transition state stabilizing residue Asp1133 converted GTFA from a mainly α1,4 to a mainly α1,6 synthesizing enzyme. Additionally, the mutation P1026V and I1029V in a region next to the nucleophile Asp1024 resulted in synthesis of a α-glucan containing only a very small

percentage of α1,4 glucosidic linkages and increased percentage of α1,6 glucosidic linkages. Thus, in order to change the linkage specificity the NNS-motif of the reuteransucrase GTFA was changed by the SEV-motif that can be also found in the GTFR glucansucrase from *S. oralis*.

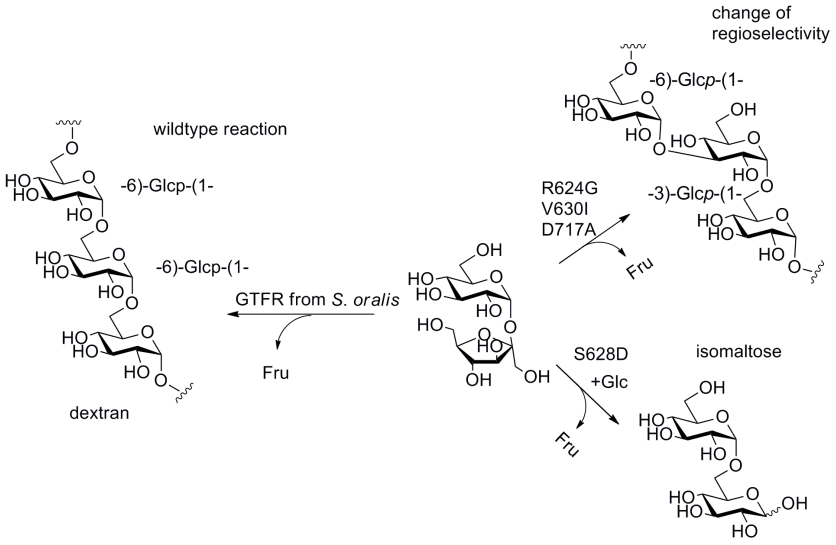

Figure 2. The triple mutant variant GTFR (R624G/V630I/D 717A) effected a switch in the region-selectivity of the glycosylation from α1,6 to predominantly α1,3 linkage formation. The polymer synthesis was knocked out by the single mutation S 628D [35].

The GTFR glucansucrase from *S. oralis* synthesizes a glucose polymer from sucrose which is mainly linked by α1,6-bonds and a minor amount of α1,3-bonds. Hellmuth *et al.* [9] have chosen the most conserved motif around the transition state stabilizer in glucansucrases for a random mutagenesis approach using this enzyme. The GTFR variant R624G/V630I/D 717A showed a drastic switch in regio-selectivity, from dextran with mainly α1,6 linkages to a mutan-type polymer with predominantly α1,3 glucosidic linkages (Figure 2). It was proposed that R624G and V630I, both mutations near the transition state stabilizer, contribute to this change. Additionally, saturation mutagenesis at serine 628 residue switched the reaction towards the synthesis of short-chain oligosaccharides. Compared to the wild type enzyme, S 628X variants exhibited tremendous changes in their short transglycosylation product spectra while polymer formation was completely stopped. The oligosaccharide yield of the prebiotic disaccharide isomaltose was dramatically increased from 1% to 47% and the yield of leucrose reached 64%. Recently, Pijning *et al.* [20] published for the first time the crystallographic structure of a glucansucrase. The structure of a 1031-residue fragment of GTF180 from *Lactobacillus reuteri* 180, both in the native state and in complexes with sucrose and maltose was described. Structural analysis, as well as docking studies allowed

concluding that the enzyme sub-sites +1 and +2 are not very specific regarding the glycosidic linkage between the sugars, and can accommodate either α1,3, α1,4, or α1,6-linked glucose residues. To summarize, the linkage specificity and the oligosaccharides *versus* polysaccharide formation ratio could be controlled and engineered by site directed mutagenesis including the knowledge of one enzyme's structural features and functionalities.

Substrate-engineering – Substrate Directed Synthesis

As previously mentioned, different strategies should be addressed in order to expand the range of tailored products to meet the required specification for a targeted application in chemical, cosmetics, food or medical fields. While protein engineering allows the synthesis of a large variety of products with specific linkages and molecular weight, substrate-engineering expands the spectrum of new glycostructures synthesized from non-natural substrates. The synthesis of glycoethers and glycopeptides offers a potential strategy for the identification of new enzyme specificities and acceptor products which could not be inferred from sequence alignment.

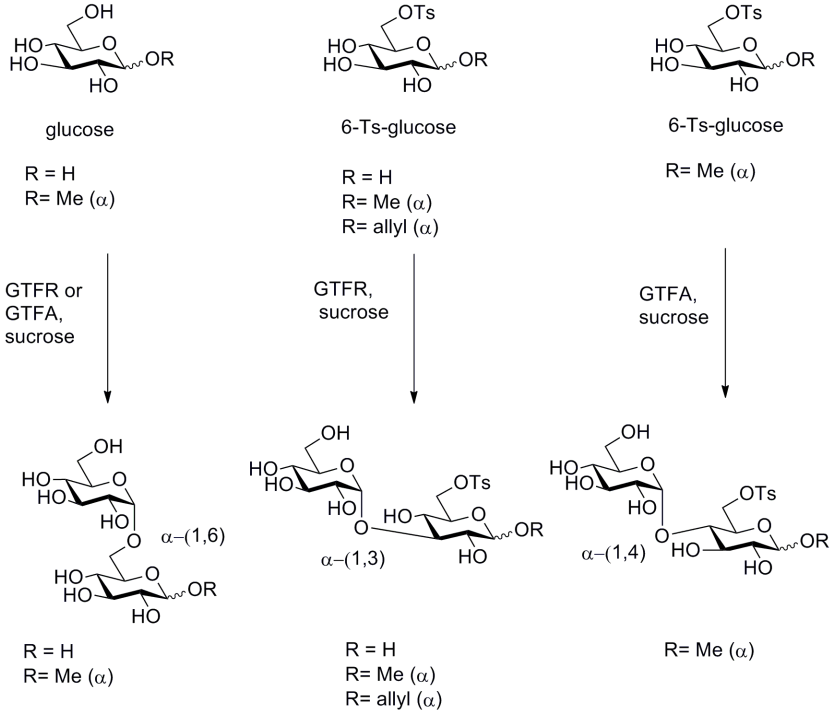

Figure 3. (a) Substrate directed synthesis addresses the linkage selectivity; **(b)** Chemical nucleophilic substitution of the tosyl group with thio-sugars yields different glycoconjugates containing thioglycosidic linkages.

Seibel *et al.* [11] demonstrated new acceptor reactions using the *S. oralis* GTFR on substrate microarrays for glycosylation of immobilized oligosaccharides. The α-glycosylation by GTFR with sucrose and different alcohols and amino acid derivatives for the synthesis of glycoethers and glycosylated amino acids was also demonstrated. These syntheses are not easy to be obtained by chemical or enzymatic synthetic methods alone. Seibel *et al.* [12] also have synthesized new sucrose analogues using the *B. subtilis* NCIMB 11871 levansucrase, which may be used for the screening of new glycosyltransferase specificities and new variants of this class of enzymes, leading to further development of novel products. Later on, Hellmuth *et al.* [10] changed the chemoselectivity of glucansucrases GTFR from α1,6- to α1,2-, α1,3- or α1,4-linked glucose residue by introducing on tosyl-residue in position 6 of the acceptor molecule glucose. The new acceptor molecule most likely adapts a different orientation in the enzymes pocket. This explains the change in the linkage type. With this approach various complex glycoconjugates containing thioglycosidic linkages with the glycopyranosides (galactose, glucose, neuraminic acid) of choice were successfully constructed (Figure 3).

Thioglycosides are tolerated by most biological systems, but are less susceptible to acid/base or enzyme-mediated hydrolysis, contrary to the naturally occurring O-glycosides [24, 25]. In addition the allyl group may be modified by ozonolysis into an aldehyde group. The modified sugar thus obtained can now be attached to aglycons, such as peptides, natural products, drugs or solid supports, such as microtiter plates for example, by reductive amination [26] allowing the investigation of protein–carbohydrate interactions. The synthesized molecules may then be used in further experiments to identify carbohydrate-lectin and -selectin interactions of biological relevance. The combination of different acceptors and enzymes as well as chemical synthesis could lead to a powerful set of tools for glycosynthesis. Those studies will be expanded in future to other glucansucrases and glycosyltransferases for the synthesis of novel structures.

Metabolic Glyco-engineering

It has been extensively reported that disorders in protein glycosylation lead to multisystemic and severe diseases. Altered glycosylation patterns have been associated with many physiological and pathological events, including cell growth, migration, differentiation, tumour invasion, host-pathogen interactions, cell trafficking, and transmembrane signalling [6]. Additionally, modified cell surfaces through deglycosylation or by a false-escorted recognition processes can also lead to an increased risk of bacterial and viral infections [27]. Sialylation is a determinant factor in pathogens and toxins binding to animal cells via cell surface sialic acids (Sias) that are α2,3-, α2,6 or α2,8-linked to the glycan chains. For example, human influenza A/B viruses prefer α2,6- linked Sias, while undergoing a switch from α2,3-linkage preference during adaptation from animals to humans [28]. Recent data indicate that the biology of sialic acids involving around 60 genes shows more than 10 uniquely human genetic changes in comparison with our closest evolutionary relatives. For

example, *N*-glycolylneuraminic (Neu5Gc) acid and *N*-acetylneuraminic acid (Neu5Ac) are the most common sialic acids found on mammalian cell types but Neu5Gc is claimed to be missing in normal human tissues [29].

Developing tools for investigating the cellular activity of glycans will help to elucidate the molecular basis for aberrant glycosylation in pathological processes including cancer. Through metabolic glyco-engineering, sugar-reporting groups can be displayed into the cell surface glycoconjugates, constituting a powerful method for imaging the localization, trafficking, and dynamics of glycans and isolating them for glyco-proteomic analysis [30 – 32]. Mahal *et al.* [30] reported a strategy for remodelling the cell surface by exploiting the substrate promiscuity of oligosaccharide biosynthetic pathways and using the natural metabolic processes of the cell to introduce a ketone group into cell surface–associated Sias residues. Authors proposed that engineered cell surfaces bearing unusual epitopes may be selective targets for drugs, radionuclides or imaging reagents. The viability of this approach was further demonstrated when engineered cells were coupled to biotin through selective conjugation to ketone groups, and selectively killed in the presence of a ricin A chain–avidin conjugate. In order to diversify the range of carbohydrates that may be displayed on cell surfaces, Oetke *et al.* [31] studied the ability of eukaryotic cells to uptake Sias from the extracellular space, finding that free sialic acid can be taken up and efficiently incorporated in a variety of human cell lines. Later on, Hsu *et al.* [32] reported the incorporation of alkynyl sugar monomers based on fucose and *N*-acetylmannosamine into fucosylated and sialylated glycans in several cancer cell lines, allowing the visualization of cell surface and intracellular glycoconjugates. The authors demonstrated that click-activated fluorogenic probes are practical tools for efficiently and selectively labelling alkynyl-modified glycans. Homann *et al.* [33] reported the synthesis and bioorthogonal metabolic incorporation of the sialic acid analogue *N*-(1-oxohex-5-ynyl) neuraminic acid (Neu5Hex) into the cell-surface glycocalyx of a human larynx carcinoma cell line (HEp-2) and its fluorescence labelling by click chemistry. The bioorthogonal modification, that is the introduction of hexyne, was carried out at the sialic acid acetyl residue at position C-5 which is reported to be prone to mammalian evolution processes.

HEp-2 cells were investigated because of their metabolic capability to incorporate 2-azidoacetylamino-2-deoxy-(1,3,4,6)-tetraacetyl-β-D-glucopyranoside (Ac4GlcNAz) by a diffusion process through the membrane of eukaryotic cells [34]. Neu5Hex was expected to enter the cell by the previously described pinocytosis processes or by a yet unknown internalization mechanism. It is proposed that Neu5Hex enters the nucleus and enhances the genetic feedback control of the GNE coding gene which blocks the synthesis of natural Neu5Ac [33] (Figure 4). The incorporation and cell surface presentation of the new substrate Neu5Hex was successful and the copper-catalysed [3+2] triazole formation (“click reaction”) proved very useful for the cell surface labelling because of its bioorthogonality (Figure 5). The metabolic labelling of human larynx carcinoma (HEp-2) could make possible the study of the influence of sialic acid in cell signalling and cell-cell interactions.

This method will provide useful information about their roles in cancer and ultimately can be potentially used for diagnostic and therapeutic purposes [32].

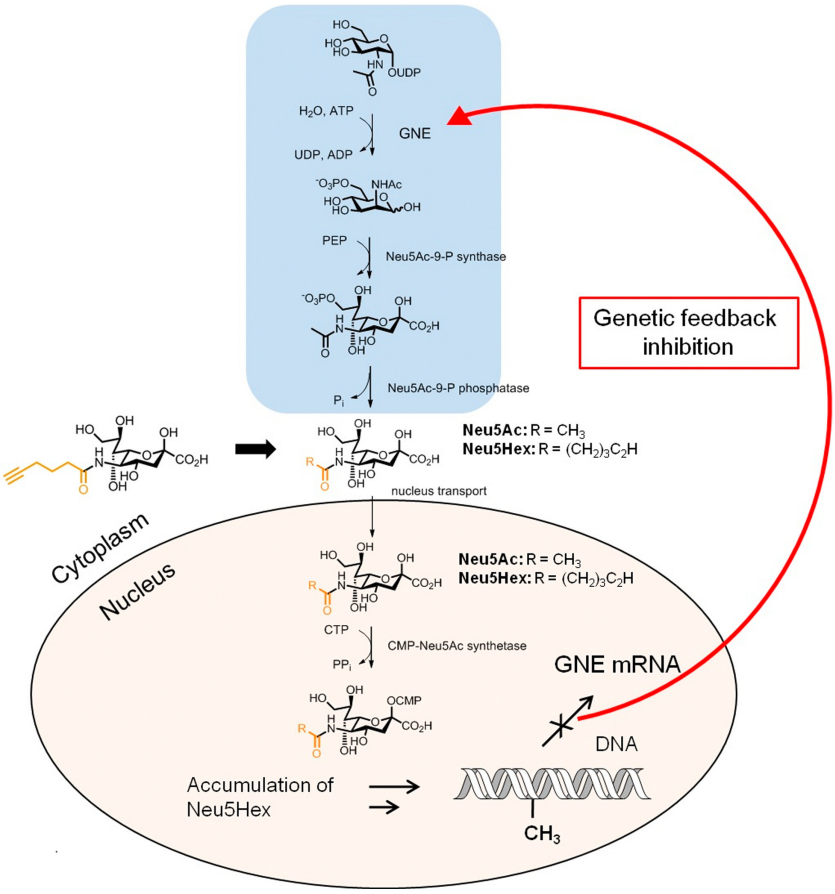

Figure 4. Metabolic pathway of Ac4GlcNAz and the genetic control of Neu5Ac synthesis by feedback inhibition [33]. The accumulation of Neu5Hex is proposed by its incubation with the target cell line as the synthesis of Neu5Ac is down-regulated. UDP: uridine diphosphate; GNE: UDP-*N*-acetylglucosamine 2 epimerase/*N*-acetylmannosamine kinase; ATP: adenosine triphosphate; PEP: phosphoenolpyruvate; CTP: cytidine triphosphate; PPi: pyrophosphate; DNA: deoxyribonucleic acid; mRNA: messenger ribonucleic acid.

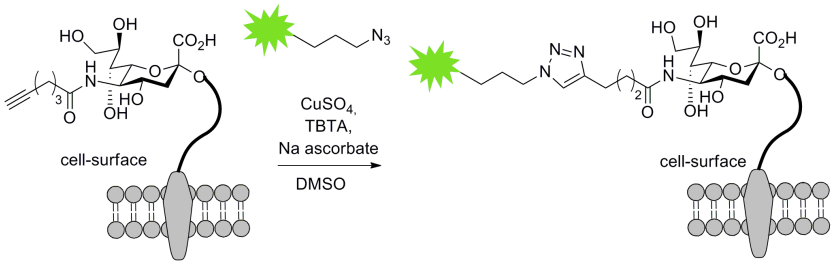

Figure 5. Labelling of alkynylated neuraminic acid by azido-fluorescein [33].

Perspectives

In spite of the information concerning glycosylation disorders, the biochemical properties, as well as the structure-function relationship of the glycosyltransferases involved in the glycosylation process are, however, hardly understood.

The study of different human glycosyltransferases is currently in progress in our working group and is intended to contribute to the basic understanding of the biochemical and structural properties of the human glycosyltransferases regarding its modulation. In this sense structural studies of eukaryotic glycosyltransferases are also needed to add valuable information leading to the development of potential inhibitors and also to allow the synthesis of glycoproteins and glycoconjugates for therapeutic applications.

Conclusion

During the last years different approaches have been attempted in order to elucidate the structural determinants directly involved in the carbohydrate active enzymes selectivity. The main goal of this search is either modifying and/or expanding the functional range of target enzymes in order to control the synthesis of their products fulfilling the needs in understanding the nature's glycosylation repertoire and providing the chemical, food, cosmetics and pharmaceutical industries with sugars.

Besides protein engineering, the search for new functionalities has been extended to different fields including solvent and substrate engineering. This review of our science and others describes the production of tailor-made oligosaccharides and polysaccharide structures with potential application in health care and food industry that have been successfully achieved by combination of different strategies. It also describes the synthesis and cell surface display of sialic acid analogues for human larynx carcinoma labelling with potential application in diagnosis.

Acknowledgment

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