Bioorganic & Medicinal Chemistry 17 (2009) 2645–2653

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Review

The effect of individual N-glycans on enzyme activity

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ARTICLE INFO

Article history:
Received 1 November 2008
Revised 11 February 2009
Accepted 13 February 2009
Available online 25 February 2009

Keywords:
N-Linked glycosylation
N-Linked glycans
Site-directed mutagenesis
Enzyme activity
Binding affinity
Protein secretion
Receptor function

ABSTRACT

In a series of investigations, N-glycosylation has proven to be a key determinant of enzyme secretion, activity, binding affinity and substrate specificity, enabling a protein to fine-tune its activity. In the majority of cases elimination of all putative N-glycosylation sites of an enzyme results in significantly reduced protein secretion levels, while removal of individual N-glycosylation sites often leads to the expression of active enzymes showing markedly reduced catalytic activity, with the decreased activity often commensurate with the number of glycosylation sites available, and the fully deglycosylated enzymes showing only minimal activity relative to their glycosylated counterparts. On the other hand, several cases have also recently emerged where deglycosylation of an enzyme results in significantly increased catalytic activity, binding affinity and altered substrate specificity, highlighting the very unique and diverse roles that individual N-glycans play in regulating enzyme function.

Contents

1. Introduction

It has been estimated that over half of all proteins found in Nature are glycosylated, with more than three quarters of these containing N-linked carbohydrates.[1] N-Glycosylation, one of the most common and yet complex forms of post-translational modification, is intricately involved in a host of cellular processes including protein folding,[2–4] protein secretion,[5] intracellular trafficking[6,7] and cell communication.[8,9] The evidence of the importance of N-glycosylation in regulating glycoprotein structure and function is overwhelming.[10–18] In general, N-linked glycans confer stability to the glycoprotein to which they are attached,[19,20] increase

Abbreviations: Ac, acetyl; ADAM10, a disintegrin and metalloprotease 10; Ala, alanine; Arg, arginine; Asn, asparagine; AVD, avidin; BSF, bovine serum fetuin; C6ST, chondroitin 6-sulfotransferase; CHO, Chinese hamster ovary; CSFV, classical swine fever virus; DPP, dipeptidyl peptidase; EL, endothelial lipase; ER, endoplasmic reticulum; Gal, galactose; GAT1, GABA transporter; Glc, glucose; GlcNAc, *N*-acetylglucosamine; Gln, glutamine; HDL, high density lipoprotein; HEK, human embryonic kidney; HL, hepatic lipase; HPC, human protein C; KSST, keratin sulfate sulfotransferase; LAL, lysosomal acid lipase; LCAT, lecithin:cholesterol acyltransferase; LDL, low density lipoprotein; LH, lutenizing-hormone; LINCL, late infantile neuronal ceroid lipofuscinosis; LPL, lipoprotein lipase; Man, mannose; OVT, ovotransferrin; Ser, serine; Thr, threonine; tPA, tissue plasminogen activator; YEI, yeast external invertase.

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doi:10.1016/j.bmc.2009.02.037

plasma residence times,[21,22] and provide steric protection from proteases and non-specific interactions.[20] Further structural roles for N-linked oligosaccharides include the maintenance of protein quaternary structure, and modification of protein binding through a host of carbohydrate-based ligand–receptor interactions.[9,20]

The modulation of protein structure and function by asparagine-linked glycosylation has been elegantly reviewed,[13,18,23] and the effects of N-glycosylation on protein secondary structure, folding, physicochemical properties, stabilization and recognition events, is well understood. In addition to these roles, recent studies have shown that N-glycosylation can also have a dramatic effect on enzyme activity, targeting and substrate specificity. In recent years a number of impressive studies have appeared involving the systematic removal of the putative N-glycosylation sites of various glycosylated enzymes in order to uncover the precise role each individual N-glycan plays in regulating enzyme secretion, activity, and substrate specificity. The key results and current trends emerging from these studies are reviewed here.

2. Deglycosylation

N-Linked glycosylation is predicted to occur at the asparagine (Asn, N) residue of the Asn-Xaa-Ser/Thr consensus sequence of a protein, where Xaa is any amino acid except proline.[24,25] Recent studies estimate that approximately 70–90% of these sequences are glycosylated.[26] N-Glycans, the biosynthesis of which has been well described in the literature,[27,28] are divided into three main types referred to as high mannose, complex and hybrid N-glycans (Fig. 1).[29] In addition to the remarkable structural complexity that these oligosaccharide chains convey to the protein,[30] further complexity arises from the ability of different N-glycans to occupy the same glycosylation sites. This gives rise to various glycoforms of a protein, each of which may have their own unique function.[31,32] Furthermore, cell-type specific expression of N-glycans can occur,[33] adding yet another layer of sophistication to the glycosylation process.[28,29]

In recent years, a number of studies have appeared involving the removal of N-glycans from proteins, either individually or in combination, in order to assess their role in enzyme secretion, stability and activity. Protein deglycosylation is typically achieved using site-directed interconversion of the asparagine (Asn, N) residue of the Asn-Xaa-Ser/Thr consensus sequence to the structurally related glutamine (Gln, Q) residue, or to other amino acids such as alanine (Ala, A). Deglycosylation can also be affected by chemical means using inhibitors of glycoprotein-processing enzymes such as tunicamycin, which blocks the first step in glycoprotein synthesis; castanospermine, which inhibits α- and β-glucosidase activity in the second step of glycoprotein synthesis; and deoxymannojirimycin or swainsonine, which block the conversion of high mannose to complex-type N-glycans.[34] Alternatively, enzymatic deglycosylation can be performed on the secreted glycoprotein using glycosidases such as PNGaseF, which cleaves between the innermost GlcNAc and Asn residues of most N-linked glycoproteins; Endoglycosidase F or H which cleave between the two GlcNAc residues of the chitobiose core leaving one GlcNAc residue attached to Asn; and/or using neuraminidase and β-galactosidase enzymes, which result in terminal trimming of complex and hybrid N-glycans (Fig. 2).[13,29]

Utilizing one or more of the deglycosylation strategies described above, the majority of cases involving removal of all putative N-glycosylation sites of a protein result in either abolished or significantly reduced secretion of the active enzyme (Section 3). On the other hand, removal of individual putative N-glycosylation sites often leads to varied effects on enzyme function and structure, in some cases markedly decreasing enzyme activity and in others significantly increasing catalytic activity and/or altering substrate specificity (Section 6).

These results are, however, often dependent on the manner in which the deglycosylated material has been prepared, with each method having its own associated drawbacks. For example, the relatively harsh conditions of chemical deglycosylation may effect enzyme secretion as well as activity; enzymatic deglycosylation may be inhibited by steric hindrance and/or the presence of certain glycan residues; and both enzymatic deglycosylation and site-directed mutagenesis have the potential to introduce a change to the tertiary structure, and consequently the function and activity of the protein.

Herein, recent protein deglycosylation experiments will be reviewed, with a particular focus on studies where individual sites were removed and the biological effect of their elimination examined. For the reasons described above, the method of deglycosylation has been highlighted for the various studies, as it should be taken into consideration that this may influence the ensuing results.

3. Effect on enzyme secretion

The fundamental role of N-glycosylation in protein folding and trafficking through the ER and Golgi is well known.[35] In the last few decades a number of protein deglycosylation studies have also

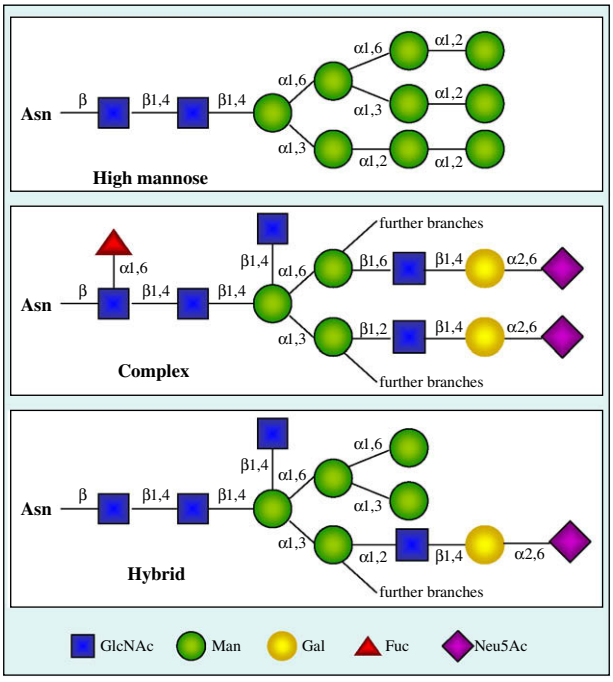

Figure 1. Schematic representation of typical high mannose, complex and hybrid type N-linked glycans.

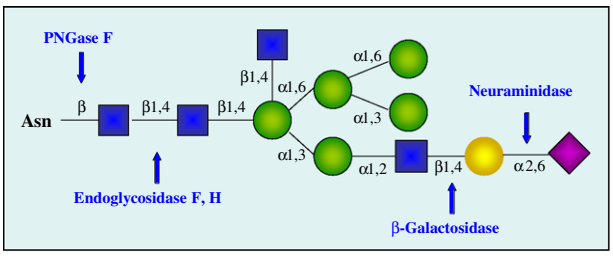

Figure 2. Schematic representation of some of the common enzymatic deglycosylation strategies used for N-linked glycoproteins.

highlighted the crucial part that N-glycosylation plays in enzyme secretion. In 1988, Hori et al. reported on site-directed mutagenesis studies showing that N-glycosylation of human renin, although not important for enzyme activity, was essential for its effective secretion,[36,37] while N-glycosylation of one of the hepatitis B virus envelope glycoproteins (M) was also shown to be important for viral protein folding and secretion.[38,39] Further examples include mammalian zinc metalloendopeptidases such as heavily N-glycosylated meprin A. Treatment with tunicamycin was found to drastically reduce meprin A secretion, whereas castanospermine and swainsonine had little effect on secretion.[40]

Human lipoprotein lipase (LPL), hepatic lipase (HL) and endothelial lipase (EL) are members of the triglyceride lipase gene family, which play a central role in lipoprotein metabolism. A series of glycosylation studies have shown that LPL bears two N-glycans at N43 and N359,[41–43] while both HL and EL are decorated by four N-glycans (HL: N20, N56, N340 and N375, EL: N62, N118, N375 and N473) with two N-glycosylation sites conserved amongst all three enzymes (Fig. 3).[44–47] Site-directed removal of the conserved N-terminal glycosylation site dramatically reduced secretion of all three lipases from mammalian cells, while mutation of the conserved C-terminal site resulted in differential effects that were largely cell-type dependent. For example, mutation of EL residue N375, did not effect secretion from COS (African green monkey kidney) cells,[45] but compromised secretion from human embryonic kidney (HEK) cells,[46] while removal of the homologous residue in HL (N375) did not effect secretion from Chinese hamster ovary (CHO) cells,[47] but reduced secretion from COS cells by 60%.[41–43]

In 2007, N-glycosylation of the membrane glycoprotein dipeptidyl peptidase IV (DPP-IV) was reported to regulate protein trafficking, stability, folding and activity.[48] Site-directed mutagenesis studies of DPP-IV using CHO cells found that when three of the eight potential glycosylation sites (N83, N686 and N319) were individually removed, the resulting deglycosylated mutants showed reduced half-lives. While deglycosylation of DPP-IV at residues N83 and N686 had little effect on enzymatic activity, removal of the glycosylation site at residue N319 resulted in the production of an inactive enzyme that was not expressed on the cell-surface, failed to dimerize and showed increased degradation.[48]

Transmissible spongiform encephalopathies are neurological diseases thought to derive from the misfolded form of the host glycoprotein prion protein. Recent studies have shown that the N-glycans of prion proteins play an important role in the folding and misfolding of the protein,[49] and may also reduce the rate of fibrillization by promoting intramolecular disulfide stability.[50] Related studies into the protein folding of the human immune CD2 cell receptor have revealed that N-linked glycosylation increases protein stability and greatly facilitates the folding process. A fully deglycosylated CD2 variant showed a fourfold reduction in folding, while enzymatic removal of the terminal sugar moieties led to the identification of the innermost trisaccharide as important for folding and stabilization of CD2.[51]

There are many more examples where N-glycosylation is reported to drastically affect the secretion of the active conformation of an enzyme including vitamin K-dependent carboxylase,[52] cryptococcal phospholipase B1,[53] autotaxin lysophospholipase D[54] and β-1,3-*N*-acetylglucosaminyltransferase.[55] It should however be kept in mind that in several of the experiments described here the impact of deglycosylation on secretion was assessed by measuring the amount of enzyme secreted into the media, and this experimental design does not differentiate between the various factors affecting secretion, such as protein folding, aggregate formation, intracellular trafficking and/or stability. Thus, before it can be determined whether (and how) glycosylation may modulate enzyme secretion, more detailed investigations are required, where the effect of deglycosylation on each of these factors is determined individually.

Furthermore, it can not be said that N-linked glycosylation is essential for protein folding and secretion in all cases, as some studies have reported a null effect after selective removal of protein N-glycosylation sites. For example, studies on the β-subunit of bovine lutenizing-hormone (LH) have shown that removal of the single N-glycosylation site increased intracellular stability, but did not affect transport and secretion of the active enzyme or LH signal transduction.[56] In the examples to follow, N-glycosylation does not necessarily affect the intracellular trafficking and secretion of a particular glycoprotein, but is found to be essential for the full complement of enzymatic activity.

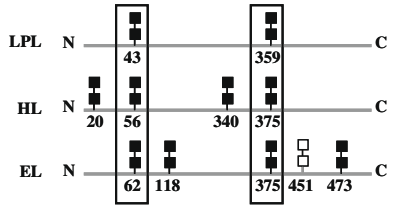

Figure 3. N-Glycosylation sites in LPL, HL and EL (non-used sites: white squares; used sites: black squares; conserved residues enclosed in boxes).

4. Effect on receptor function

The remarkable structural complexity of oligosaccharides means that they can convey incredibly large amounts of information leading to highly specific interactions.[30] The role of N-linked glycans on proteins facilitating recognition events has been well described in the literature.[18] A selection of recent examples illustrating the effect of systematic deglycosylation on receptor function is presented here.

Reduced receptor–ligand affinity has been described for the murine pregnancy-specific glycoproteins and their CD9 receptor[57] and the porcine growth hormone receptor.[58] In both cases, the decrease in receptor–ligand binding affinity typically becomes more pronounced with increasing deglycosylation, such that the fully deglycosylated enzymes show only minimal activity or binding.[59] Further investigation into the role of N-glycosylation on the assembly and function of the heteromeric *N*-methyl-D-aspartate receptor in HEK293 cells, found that tunicamycin treatment resulted in significantly decreased binding of the receptor, accompanied by a fourfold reduction in the dissociation constant.[60] While, in 2005 Fan and co-workers reported studies on the GABA transporter GAT1, which contains three putative N-glycosylation sequences. Site-directed mutagenesis of GAT1 in CHO cells produced glycan-deficient mutants that exhibited significantly diminished protein stability and trafficking to the plasma membrane, and markedly reduced GABA-uptake activity, whereas 1-deoxynorjirimycin treated GAT1 mutants revealed that terminal trimming of the N-glycans affected activity but not secretion and trafficking.[61]

Recent studies on the human relaxin receptor (RXFP1), have revealed six N-glycosylation sites (N14, N105, N242, N250, N303 and N346), all of which are occupied. Site-directed mutagenesis in HEK293 cells was used to produce a range of single-, double- and multiple glycan deficient mutants, which revealed that glycosylation did not effect relaxin binding, but was important for cell surface expression and cAMP signalling, with the N303-glycan playing the greatest role.[62] N-glycosylation was also found to be important for the trafficking of other G-protein-coupled receptors such as the follicle stimulating hormone receptor,[63] human thyrotropin receptor[64] and luteinizing hormone receptor.[65]

As reported in 2007, N-glycosylation has been found to play an integral role in the survival and maturation of classical swine fever virus (CSFV), whereupon heavily N-glycosylated viral glycopro-

teins form active homo and heterodimer complexes that are an important part of the viral life cycle. For certain viral glycoproteins such as E2, tunicamycin treatment blocked protein secretion, dimer formation and markedly inhibited CSFV spread in swine kidney cells, raising the possibility that glycosylation inhibitors could be used to reduce CSFV progression.[66]

5. Effect on enzyme thermostability

N-Linked glycosylation is known to improve protein stability,[19,20] increase circulation in plasma[21,22] and provide steric protection of susceptible sites such as hinges and linkers from attack by proteases and non-specific interactions.[20] In earlier sections, the stabilization of DPP-IV,[48] human CD2[51] and GAT1[61] through N-linked glycosylation was described. Other examples include an investigation into the role of N-glycosylation in the stability of the proteinase cathepsin E via site-directed mutagenesis in rat kidney cells, which has found that removal of the putative N-linked glycosylation sites at N73 and N305 results in reduced stability towards heat and acid.[67]

A detailed study by Giartosio and co-workers[68] on the influence of glycosylation on the thermostability of a range of glycoproteins including four N-linked examples, yeast external invertase (YEI), bovine serum fetuin (BSF) and chicken egg white avidin (AVD) and ovotransferrin (OVT), found that deglycosylation using exo- and endoglycosidases resulted in decreased protein thermostability for the heavily glycosylated YEI and BSF, but did not significantly affect the thermostability of the less glycosylated AVD and OTF. Deglycosylation of YEI and BSF did not appear to alter protein secondary structure, but did lead to poorer reversibility of the thermal denaturation process and a higher tendency to aggregate during thermal inactivation compared to their glycosylated counterparts. These results suggest that in addition to stabilizing the protein conformation, glycosylation may also prevent unfolded or partially folded proteins from aggregating, and that both of these elements are closely linked to the degree of glycosylation.

Recent studies on the role of N-glycosylation of the subunits of oligosaccharyl transferase from *Saccharomyces cerevisiae*, have revealed that three of the essential gene products, Ost1p, Wbp1p and Stt3p, are N-glycosylated proteins.[27,69] Site-directed mutagenesis studies showed that four N-glycosylation sites in Ost1p and two in Wbp1p were occupied. These sites were not conserved between subunits and their removal affected neither enzyme secretion nor activity. On the other hand, the subunit Stt3p contained two conserved N-glycosylation sites at N535 and N539, with mass spectrometric analysis identifying only single occupation by Man(8)GlcNAc(2) at N539. Point mutations of these two sites resulted in temperature sensitive oligosaccharyl transferase mutants, which did not exhibit any growth defects.[69]

Studies on the removal of N-linked sugar residues from glucoamylase from the fungi *Aspergillus niger* resulted in the exposure of hydrophobic regions of the protein, thereby leading to diminished thermostability, and increased enzyme flexibility and aggregation,[70] with similarly decreased secretion and thermostability observed for glucoamylase from *Aspergillus awamori*,[71] while for α-amylase from *Aspergillus oryzae*, N-glycosylation was found to have no observable effect on enzyme secretion, activity or thermostability.[72] In complementary studies, artificially introducing new glycosylation sites into glucoamylase from *A. awamori* has been shown to increase its thermostability.[73]

6. Modulation of enzyme activity/specificity

There are a small number of N-glycosylated glycoproteins where mutational studies have been unable to attribute any effect of deglycosylation on enzyme secretion, activity and stability including glutaminyl cyclase,[74] and 11 β-hydroxysteroid dehydrogenase.[75] However, the majority of studies have shown significant and often drastic effects to an enzyme resulting from removal of one or more of its N-glycosylation sites.

6.1. Decreased enzyme activity upon deglycosylation

In the majority of cases, removal of one or more N-glycans from an enzyme results in significantly reduced catalytic activity relative to that of the wild-type enzyme. For example, in studies into the N-glycosylation site occupancy and its effect on the enzymatic activity of human acid β-glucosidase, the five potential glycosylation sites were each eliminated to generate singly glycan-deficient mutants at residues N19, N59, N146, N270 and N462 in insect Sf9 and COS-1 cells. Deglycosylation at residue N19 markedly reduced the catalytic activity of the enzyme, whereas removal of the N-glycans at N59, N270 and M462 had little effect on activity. Additionally, the N146Q-glycan deficient mutant showed reduced thermostability, and residue N462 was determined to be unoccupied. The effect of altering residue N19 was also dependent on the newly introduced residue, with N19Q, N19E, and T21G mutants showing a 60-, 30- and 3-fold reduction in enzymatic activity respectively, and the N19D mutant showing a threefold enhanced activation by phosphatidylserine, highlighting the important role that individual glycans play in generating and maintaining a catalytically active enzyme.[76]

In 1996, the lysosomal murine acid sphingomyelinase was reported to be N-glycosylated at residues N84, N173, N333, N393, N518 and N611. Site-directed mutagenesis studies revealed that alteration of the glycosylation sites at N333/N393 and N518 reduced the enzyme activity by 60% and 80%, respectively, while removal of the remaining N-glycans had little effect on activity. The markedly reduced enzyme activity was attributed to poor enzyme stability for the deglycosylated N333 and N393 mutants and protein misfolding for the N518 mutant.[77]

In a *pas de deux* of enzyme glycosylation, glycosyltransferases, the enzymes responsible for attaching sugar residues to other proteins, are themselves glycosylated. In 1995, a key glycosyltransferase in glycoconjugate biosynthesis, β-1,4-*N*-acetylgalactosaminyltransferase, was reported as containing three potential N-glycosylation sites, each of which was removed through site-directed mutagenesis. It was found that elimination of a single N-glycosylation site resulted in an appreciable reduction in enzyme activity, with the decrease in activity proportional to the extent of deglycosylation, and the fully deglycosylated mutant showing just 10% residual activity.[59]

In further studies on sugar processing enzymes, site-directed removal of the five glycosylation sites of human β-1,3-*N*-acetylglucosaminyltransferase II (GlcNAcT-II), a key enzyme involved in the biosynthesis of N-linked oligosaccharides, revealed that glycosylation at N219 is essential for enzyme activity, while sugar residues at N127 and N219 are required for effective enzyme secretion.[55] Similarly, N-glycosylation of rat *N*-acetylglucosaminyltransferase III (GlcNAcT-III) was found to be essential for its activity with point mutations of the glycosylation sites at N243, N261 and N399 in COS-1 cells, producing mutants with reduced enzyme activity relative to wild-type, with the most pronounced effect obtained with the N243 glycan-deficient mutant.[78] The reduced activity was commensurate with the number of glycosylation sites available, and elimination of all sites generated an inactive enzyme (Fig. 4). All glycan-deficient mutants from this study also showed poor retention in the Golgi.

In 2000, systematic mutational studies revealed that C-terminal N-glycosylation of the human α-1,3/4-fucosyltransferases III, V and VI (hFucT-III, -V and -VI) responsible for fucosylation of cell surface

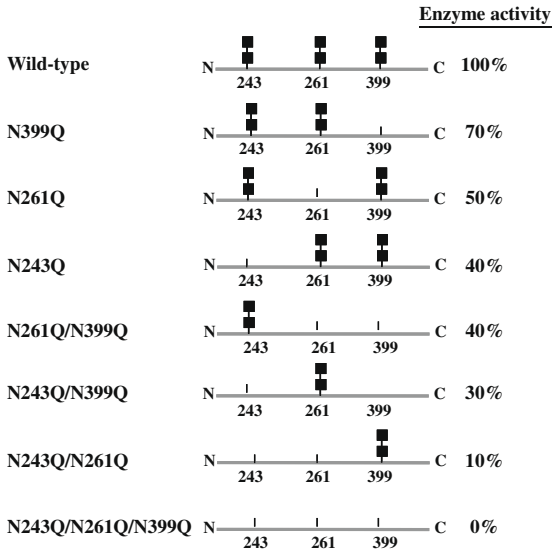

Figure 4. N-Glycosylation sites in rat *N*-acetylglucosaminyltransferase III (GlcNAcT-III) and the effect on activity upon their site-directed removal.

glycoconjugates, is also essential for full enzyme activity.[79] While tunicamycin treatment abolished the enzyme activity of hFucT-III, site-directed removal of the two conserved glycosylation sites (hFucT-III: N154/185; hFucT-V: N167/198 and hFucT-VI: N153/184) and expression in COS-7 cells produced glycan-deficient mutants with differential effects on enzyme activity, all of which were reduced relative to the corresponding wild-type enzyme. Further studies on rat α-1,3-fucosyltransferase IV (rFucT-IV) resulted in the production of a glycan-free rFucT-IV mutant that was devoid of enzymatic activity. Site-directed mutation of the individual glycosylation sites revealed that glycosylation at N218 is critical for full activity, while glycosylation at N117 plays a minor role, with the two mutants showing reduced enzyme activity of 64% and 5%, respectively. N-glycosylation, which did not appear to affect intracellular trafficking, was thought to either play a direct role in the enzyme function or an indirect role in maintaining the active enzyme conformation.[80]

In recent studies from 2004, human sialyltransferase, ST3GalI, was found to contain four putative N-glycosylation sites, which did not appear to be associated with enzyme activity, but were required for proper folding and trafficking of the enzyme.[81,82] On the contrary, earlier studies using CHO cells had found that transferase activity in α-2,6-sialyltransferase was linked to glycosylation, with the penultimate galactose residue of the complex N-glycans being particularly important.[83] Treatment of mouse sialyltransferase II with the glycosylation inhibitor castanospermine resulted in a 75% reduction in specific activity of the enzyme expressed in neuroblastoma N108-15 cells.[82] Similar effects on enzyme secretion and activity have been reported for other glycosyltransferases including β-1,3-galactosyltransferase[84] and GlcNAc-6-sulfotransferase-1.[85] Unravelling the role of N-glycosylation on the enzyme activity and intracellular trafficking of glycosyltransferases is an area that perfectly highlights the complexity of the glycosylation process.

Further examples of systematic enzyme deglycosylation were reported in 2001, with N-glycosylation of the glycine transporters GLYT1 and GLYT2 reported to play an important role in the targeting and activity of both enzymes. Enzymatic and chemical deglycosylation was found to reduce transport activity of both enzymes by around 40%, while mutational studies of the four potential glycosylation sites of GLYT1 (N169, N172, N182, N188) showed decreased transport activity in line with decreasing availability of glycosylation sites, and removal of the four glycosylation sites of GLYT2 (N345, N355, N182, N188) resulted in an inactive protein that was intracellularly retained. The glycan deficient GLYT1 mutant also showed reduced trafficking to the plasma membrane.[86,87]

Human gastric lipase plays a key role in the digestion of triacylglycerols. Single point mutations of its potential N-glycosylation sites (N15, N80, N252 and N308) revealed that glycosylation at N308 is essential for full enzyme activity as the deglycosylated mutant showed only half the level of specific activity of the wild-type enzyme, while the absence of the remaining glycosylation sites did not affect enzyme activity.[88]

E-cadherin plays a key role in mediating calcium-dependent cell–cell adhesion between epithelial cells and is known to possess tumour suppressor activity. A recent study involving site-directed mutagenesis of each of its four N-glycosylation sites at N554, N566, N618 and N633 in a human breast carcinoma MDA-MB-435 cell line, have identified distinctive functions for the individual N-glycans. For example, the N633-glycan is critical for enzyme stability, while N-gylcosylation at N554 and N566 is essential for the tumour-suppressive role of E-cadherin in cell cycle progression, thought to operate through the extracellular signal-regulated protein kinase signaling pathway.[89]

Mounting evidence suggests that N-linked glycosylation may regulate β1 integrin function, based on enzymatic and chemical deglycosylation studies showing altered integrin conformation and ligand-binding activity, and numerous examples of altered β1 integrin glycosylation based on changes in cell phenotype.[90] Earlier studies involving tunicamycin treatment revealed that integrin N-glycosylation is essential for normal cell adhesion, and introduction of an artificial glycosylation site at residue #333 in the β1 integrin subunit, followed by expression in CHO-K1 cells induced the α5β1 integrin to adopt an active conformation. However, further detailed mutational studies are required to elucidate the role of the various glycans, and the occupancy of the twelve potential N-glycosylation sites remains to be determined.

Late infantile neuronal ceroid lipofuscinosis (LINCL) is caused by the deficiency of the CLN2 protein, lysosomal tripeptidyl peptidase-I. Site-directed conversion of Asn to Ser at one of the potential N-glycosylation sites (N286S) was performed in HEK293 cells to investigate the effect of this mutation on enzymatic activity and intracellular targeting, as this mutation has been found in two LINCL patients. The glycan-deficient mutant CLN2 protein showed a reduced rate of expression relative to wild-type and was devoid of enzymatic activity.[91]

In 2005, lysosomal acid lipase (LAL), a hydrolase that plays a key role in lipid metabolism, was reported as having six N-glycosylation sites, along with one O-glycosylation site. Systematic mutagenesis of each N-glycosylation site in baculovirus-infected *Spodoptera frugiperda* cells gave twelve singly and doubly glycan-deficient LAL mutants. Two of the singly deglycosylated mutants, N134Q and N246Q, were devoid of lipolytic activity and were not secreted, and all double-mutants lacking glycosylation at N9 displayed reduced activities, while the remaining single and double mutants showed comparable activity to the wild-type enzyme.[92] The role of individual N-glycans in the formation and function of a wide number of other lipolytic enzymes have been reported earlier, including gastric lipase where removal of individual N-glycosylation sites did not affect enzymatic activity[88] and human acid ceramidase in which three of the six potential N-glycosylation sites were found to be essential for activity.[93]

A highly glycosylated serine protease, BJ-48, obtained from the venom of the snake *Bothrops jararacussu*, was treated with PNGase F and neuraminidase to produce variably glycosylated derivatives which showed lower thermostability and reduced active site accessibility.[94] While recent tunicamycin-based inhibition studies on quail Sulf1, a heparin sulfate endosulfatase that regulates 6-O-sul-

fation of cell-surface heparin sulfate chains, have shown the Sulf1 contains multiple N-glycans, which are important for enzyme secretion, activity, heparin binding and membrane targeting.[95] In site-directed mutagenesis studies using HEK293 cells, it was proposed that seven of the eleven predicted glycosylation sites were occupied and the authors of the work raised the possibility that the N-glycans may have the specific role of 'presenting the heparin substrates to the catalytic site'.

In studies in 2008, the type I transmembrane glycoprotein, ADAM10 (disintegrin and metalloprotease 10), which cleaves plasma membrane proteins, was found to contain high-mannose and complex-type glycans at N267, N278, N439 and N551 that are essential for its processing, localization and catalytic activity.[96] Mutational studies showed that the T280A glycan-deficient ADAM10 mutant possessed minimal enzymatic activity and was retained intracellularly, while another mutant, S441A displayed a higher susceptibility to proteolysis. Removal of the N-glycosylation sites at N267, N439 and N551 was found to reduce, but not completely abolish, enzyme activity.

Other recent studies, such as those on the pro-atrial natriuretic peptide converting enzyme, corin, indicate that N-glycosylation may regulate enzyme cell-surface expression and activity. Deglycosylation of corin by tunicamycin treatment and expression in HEK293 cells, revealed that N-glycosylation is required for cell-surface expression and activity, with markedly reduced peptide cleavage seen for the deglycosylated variant.[97] While recent site-directed mutagenesis studies of chicory (*Cichorium intybus*) fructan 1-exohydrolase have suggested that N-glycosylation in the vicinity of the proposed inulin binding cleft may block access to the inulin substrate, while other inulin-degrading enzymes have an open cleft configuration, which allows inulin binding and subsequent degradation.[98]

There are a plethora of other examples where deglycosylation of a glycoprotein, whether chemical or enzymatic, leads to a significant reduction in protein function, binding, and/or enzymatic activity including steroid sulfatase,[99] heparan N-sulfatase[100] and UDP-glucuronosyltransferase.[101] In most cases, the decreased activity is attributed to an assumed destabilization of the active conformation of the enzyme upon deglycosylation, however, more detailed mechanistic studies on the precise role of the N-glycans beyond just conferring stability are still needed.

6.2. Increased enzyme activity upon deglycosylation

It is rare to observe an increase in enzyme activity upon removal of a single N-glycosylation site. The majority of glycoprotein deglycosylation experiments show markedly reduced protein secretion, function, binding and/or enzymatic activity. The most well known exception is the increased activity of ribonuclease (RNase) A, an unglycosylated variant of RNase B, where the carbohydrate chains, although contributing to the thermostability of the enzyme,[102] also sterically impede the oligomerization process.[31,103] Another famous example is that of tissue plasminogen activator (tPA), a serine protease that converts plasminogen into plasmin, which exists as two glycoforms: type I bearing three N-linked glycans at N117, N184 and N448, and type II with two N-glycans at N117 and N448. The plasminogen substrate also exists in two forms, type 1 containing both an N- and O-linked glycan and type 2, carrying only an O-linked glycan. The combination of type II tPA with type 2 plasminogen showed a twofold higher lysis activity compared to the more heavily glycosylated type I tPA with type 1 plasminogen.[104]

In some of the early studies showing increased activity upon deglycosylation, human protein C (HPC) was reported to contain four putative N-glycosylation sites (N97, N248, N313 and N329), with each appearing to play a distinct role in the function of the enzyme. Point mutations and subsequent expression in human HEK293 and hamster AV12-664 cells revealed that elimination of the glycosylation site at N97 significantly affected HPC secretion, while deglycosylation at N248 disrupted intracellular processing. Removal of the remaining glycosylation sites of HPC resulted in a 2–3-fold increase in anticoagulant activity due to an enhanced affinity of thrombin for Q313, raising the possibility that HPC efficiency could be improved by glycan alterations.[105]

The murine monoclonal antibody M195, which binds to the CD33 antigen, is currently being investigated for the treatment of myeloid leukemia. A humanized M195 antibody, where the N73 putative glycosylation site was removed, showed a markedly increased binding affinity for the CD33 antigen compared to the wild-type antibody. Similar results were obtained when the analogous site was also removed from the murine antibody, raising the possibility that altering the carbohydrate chains of antibodies showing promising therapeutic results may be a way of increasing their affinity.[106]

Lecithin:cholesterol acyltransferase (LCAT), a plasma protein responsible for the formation of cholesteryl ester in human plasma, contains four sialylated triantennary and/or biantennary complex N-glycans at residues N20, N84, N272 and N384, along with two occupied O-glycosylation sites.[107–110] In one of the early examples of deglycosylation significantly increasing catalytic activity, Pritchard and co-workers reported a twofold increase in LCAT activity when the C-terminal N-glycosylation site (N384) was eliminated by site-directed mutagenesis.[111] The proposed protein structure for LCAT places this particular glycosylation site adjacent to the catalytic triad, raising the possibility that the N384-glycan regulates accessibility of the phospholipid acyl chains of the substrate to the enzyme active site through steric hindrance and/or other mechanisms.[111,112]

Furthermore, removal of LCAT's remaining N-glycans (N20, N84, N272) decreased enzyme activity by 18%, 82% and 62%, respectively, with the N20 mutant showing a greater reduction in activity towards high density lipoprotein (HDL) and the N384 mutant displaying increased activity towards LDL,[113] while removal of the N272-glycan converts the protein into a phospholipase.[114] In other experiments involving neuraminidase digestion of plasma (p) and recombinant (r) forms of human LCAT, the desialylated pLCAT and rLCAT showed decreased activity of 23% and 10% respectively towards HDL, but increased activity towards a liposome substrate. Further studies on the activity of LCAT obtained from various sources (e.g., HepG2, SF21 and CHO cells) found that the N-glycan structure, enzyme activity and substrate specificity were all cell-type dependent,[115] providing further evidence that N-glycosylation may confer substrate specificity to LCAT.[108,110]

A further example where N-glycosylation is shown to be a key determinant of both enzyme activity and substrate recognition is that of endothelial lipase (EL), a plasma glycoprotein responsible for the hydrolysis of phospholipids in HDL. EL contains five putative glycosylation sites, of which four (N62, N118, N375 and N473) are occupied by complex-type N-glycans (Fig. 3) that are known to influence both its secretion and activity.[45] Recent studies examining the effect of N-glycosylation on EL-mediated phospholipid hydrolysis in a range of HDL substrates containing different apoplipoproteins (e.g., apoAI, apoE2, apoE3 and apoE4), have found that removal of the individual glycosylation sites at N118, N375 or N473 reduced the activity of each mutant to less than half that of wild-type EL for all substrates, with HDL containing apoE3 showing the greatest reduction in activity.[46] However, the most striking results were obtained upon elimination of the N-terminal glycosylation sequence (N62), which resulted in a 3–6-fold increase in activity, with the greatest enhancement observed for apoE2-containing HDL (Fig. 5). These results, supported by earlier studies showing a trend towards increased lipolytic activity upon removal

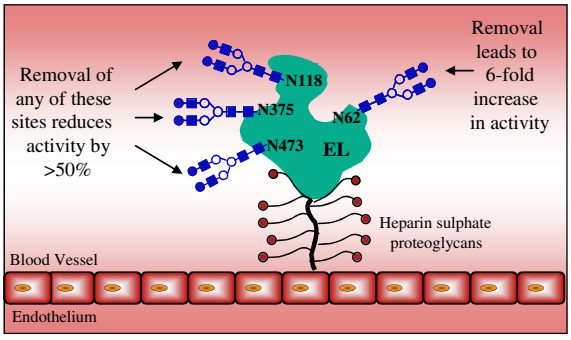

Figure 5. Schematic representation of the effect of removal of the occupied N-glycosylation sites in endothelial lipase (EL) on enzyme activity.

of the N62-glycan,[45] and other studies showing a fivefold increase in LDL hydrolysis with an N118 glycan deficient EL mutant,[116] provide the first examples where the removal of a single N-glycosylation site results in such dramatic increases in enzymatic activity.[46] They also serve to highlight the role of glycosylation in regulating substrate specificity and phospholipase activity in EL, possibly through sterically impeding access of the phospholipid acyl chains of particular substrates to the active site.

In related studies on the N-glycosylation of LPL and HL, mutation of the conserved N-terminal glycosylation site resulted in lower enzyme activity, primarily due to the markedly reduced or abolished secretion of the enzyme.[42,117] However, removal of the homologous conserved site (N57) in rat HL expressed in *Xenopus* oocytes, led to the production of a poorly secreted lipase, which was 2.7 times more active relative to the same protein mass of wild-type.[44] The structural homology model of human LPL[118–120] based on the X-ray crystal structure of human pancreatic lipase,[121,122] places the conserved N43-terminal glycan of LPL near the catalytic site.[118] Human EL shows a high degree of sequence homology (45%) with human LPL, raising the possibility that the N62-glycan of EL is also close to the catalytic site. As proposed for LCAT, it may be that N-glycans in the vicinity of an enzyme's active site, as proposed for the N64-glycan of human EL, may impede access of the substrate to the active site, such that their removal results in increased enzymatic activity. However, further X-ray crystallographic studies are required to test this hypothesis.

Further examples of increased activity upon deglycosylation include the human respiratory syncytial virus fusion protein, which was reported in 2001 to contain six N-glycosylation consensus sequences, of which three are occupied (N27, N70 and N500). Generation of a series of deglycosylated derivatives by site-directed interconversion of Asn residues to Gln revealed that glycosylation does not affect transport to the plasma membrane. However, elimination of the N70 glycan did result in a 40% increase in fusion activity, while removal of both N-glycans at residues N27 and N70 halved fusion activity. This activity was further reduced to about 10% upon removal of the glycosylation sites at N500, N27/500, and N70/500, and essentially abolished upon removal of all three glycosylation sites.[123]

Chondroitin 6-sulfotransferase-1 (C6ST-1), a glycoprotein involved in the sulfation of chondroitin and keratan sulfate, referred to as C6ST and KSST activity, has six putative N-glycosylation sequences at N63, N74, N96, N250, N413 and N457. Tunicamycin treatment of the enzyme was found to abolish production of active C6ST-1, while treatment with N-glycosidase F led to increased C6ST activity and decreased KSST activity. Selective removal of the N250 or N457-glycans also reduced production of active C6ST-1, while removal of the N413 glycan resulted in significantly decreased KSST activity.[124]

N-Glycans have also been found to regulate signal transduction by modulating receptor functions. Studies reported earlier this year on ErbB3, a member of the epidermal growth factor receptor family, involving site-directed mutation of each of the ten potential N-glycosylation sites and expression of the mutants in CHO cells, have identified that elimination of the N418 glycosylation site results in autodimerization and increased heterodimer formation. When the N418 glycan deficient mutant was co-expressed with ErbB2, it increased downstream signaling, anchorage-independent cell growth and tumour growth in athymic mice, revealing a very specific role for this particular N-glycan in regulating ErbB signaling.[125]

As N-glycosylation typically imparts improved physical, chemical and biological properties to a protein, in recent years researchers have artificially glycosylated (or glycoengineered) a range of proteins using various strategies, in order to examine the consequences of introducing new carbohydrate chains into a protein.[126–128] One example is that of microsomal cytochrome P450c17, a key enzyme involved in converting pregnenolone and progesterone into C19 steroids through its 17α-hydroxylase and 17,20-lyase activities. In this case, site-directed mutation of an Arg into an Asn residue at position 200 introduced a new potential N-glycosylation site, which resulted in increased reactivity towards pregnenolone relative to the wild-type enzyme. The glycosylated mutant was also accompanied by a reduction in 17,20-lyase activity towards 17α-hydroxyprogesterone, indicating that glycosylation can also modify substrate specificity in this particular system.[129] In another study, new glycosylation sites were introduced into the serine protease subtilisin *Bacillus lentus* using site-directed mutagenesis, to generate 48 glycosylated subtilisin mutants enzymes, with 22 of the mutants displaying increased activity towards the esterase substrate succinyl-Ala-Ala-Pro-Phe-*S*-benzyl up to 1.9-fold over the wild-type enzyme, and all 48 of the mutant enzymes showing a higher ratio of amidase to esterase activity.[130]

Further examples of artificially glycosylated proteins include eel calcitonin derivatives bearing new complex type N-glycans, which showed effects ranging from hindered receptor binding to enhanced hypocalcemic activity, dependent on the glycosylation site and N-glycan structure.[131] Furthermore, a range of therapeutic proteins have been glycoengineered to increase their carbohydrate content including darbepoetin, a hyperglycosylated analogue of erythropoietin containing two additional N-glycosylation sites and exhibiting a threefold increase in serum half-life and increased *in vivo* activity over recombinant erythropoietin.[22]

A vast number of studies have shown that individual N-glycans have very specific and diverse effects on enzyme activity, and although the majority of cases show that their removal leads to reduced activity, several examples are emerging where enzyme deglycosylation enhances activity and binding affinity and leads to altered substrate specificity.

7. Conclusions and future directions

In 1995, Dwek and co-workers described how N-glycosylation could modify the functional activity of an enzyme by increasing protein rigidity and stability as in the case of RNase B, or by sterically hindering substrate binding and access to the active site, as found for tPA,[132] and also reported that N-glycosylation typically downregulates enzyme activity and signalling,[19,132] providing a means for a protein to modulate its activity. Herein, there are a plethora of examples where protein deglycosylation, whether chemical or enzymatic, leads to a marked reduction in protein function, binding or catalytic activity. As N-linked glycans are often essential for maintaining the active conformation of an enzyme, the decreased activity observed upon deglycosylation is often

attributed to an assumed destabilization of the active conformation of the enzyme. However, further studies are needed to fully elucidate the role of N-linked glycans in these processes.

On the other hand, there are also a growing number of studies where removal of N-linked glycosylation of a protein leads to increased enzymatic activity, binding affinity and altered substrate specificity. Furthermore, in several of the examples described here, the N-linked glycans deemed essential for enzyme activity are located in the enzyme domain containing the active site,[76,80,133] raising the possibility that individual N-glycans may function directly in the catalytic activity of the enzyme. In some cases, N-glycosylation appears to act as the gatekeeper of the active site, regulating access to specific substrates. Whether this is a purely steric interaction or a more precise sugar-mediated ligand–receptor interaction will require further investigation.

Targeting glycosylation is a new therapeutic strategy in the treatment of disease[134] with increasing evidence of its important role in regulating immune function,[135,136] and in the development and progression of cancer,[137,138] inflammation,[139] muscular dystrophy,[140] Alzheimer's disease[141] and a range of congenital disorders.[142] Naturally occurring mutations of protein N-glycosylation sites in various disease states may have significant impacts on enzyme activity and thus defective enzyme glycosylation is also gaining importance as a diagnostic marker for disease.[28,139,143,144] Simply measuring levels of protein transcription and/or abundance in patients is insufficient, as post-translational events such as N-glycosylation often regulate enzyme activity and/or substrate specificity, and therefore may also play a key role in disease progression.[138]

Substantial achievements have been made in the last few decades in elucidating the role of N-glycans in enzyme secretion, stability and intracellular trafficking. Great advancements have also been made in characterizing the function of N-glycans in regulating enzyme activity, targeting and substrate specificity, yet several questions still remain. How exactly does N-linked glycosylation fine-tune enzyme activity and targeting? Can N-glycosylation function as the gatekeeper of the active site, regulating access to specific substrates? In many cases the functions of the individual N-glycans of proteins remain unknown and a complete understanding of N-glycan heterogeneity requires further investigation. Much more is needed before we can fully understand the role of N-glycosylation in enzyme chemistry and biology, but answering these important questions will enable us to diagnose diseases early, to develop targeted therapeutics and to engineer new glycoproteins for the future.

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