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Molecular mechanisms of *O*-GlcNAcylation

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Protein glycosylation with *O*-linked *N*-acetylglucosamine (*O*-GlcNAc) is a reversible post-translational modification of serines/threonines on metazoan proteins and occurring with similar time scales, dynamics and stoichiometry as protein phosphorylation. Levels of this modification are regulated by two enzymes—*O*-GlcNAc transferase (OGT) and *O*-GlcNAc hydrolase (OGA). Although the biochemistry of these enzymes and functional implications of *O*-GlcNAc have been studied extensively, until recently the structures and molecular mechanisms of OGT/OGA were not understood. This review covers a body of recent work that has led to an understanding of the structure of OGA, its catalytic mechanism and the development of a plethora of different inhibitors that are finding their use in cell biological studies towards the functional implications of *O*-GlcNAc. Furthermore, the very recent structure determination of a bacterial OGT orthologue has given the first insights into the contribution of the tetratricopeptide repeats (TPRs) to the active site and the role of some residues in catalysis and substrate binding.

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Introduction

O-GlcNAc was discovered more than 20 years ago [1], breaking the dogma that protein glycosylation was restricted to the endoplasmic reticulum, Golgi apparatus, cell surface and extracellular matrix [2]. *O*-GlcNAc was found instead in the nucleoplasm, cytoplasm and to some extent in the mitochondria. The modification was found to occur on serine/threonine residues and was not further glyco-decorated. So far *O*-GlcNAc has been found in all metazoans studied. Evidence exists for the association of *O*-GlcNAc and neurodegenerative diseases, cancer and diabetes [3]. Thus, it has been proven to be essential for neuronal function and survival in mice [4] and a correlation between the variations of *O*-GlcNAc levels (particularly on the neurofibrillary tangle protein tau) and Alzheimer's disease has been suggested [4,5]. There is also considerable evidence linking increased levels of *O*-GlcNAc to the development of insulin resistance and diabetic complications, such as hyperleptinemia, cardiomyocyte apoptosis, hypertrophy and arteriosclerosis [3]. In a very recent study, Yang *et al.* [6•] describe that human OGT (hOGT) impairs the expression of insulin-responsive genes and causes insulin resistance and dyslipidaemia through *O*-GlcNAcylation of the key signalling kinase AKT (PKB).

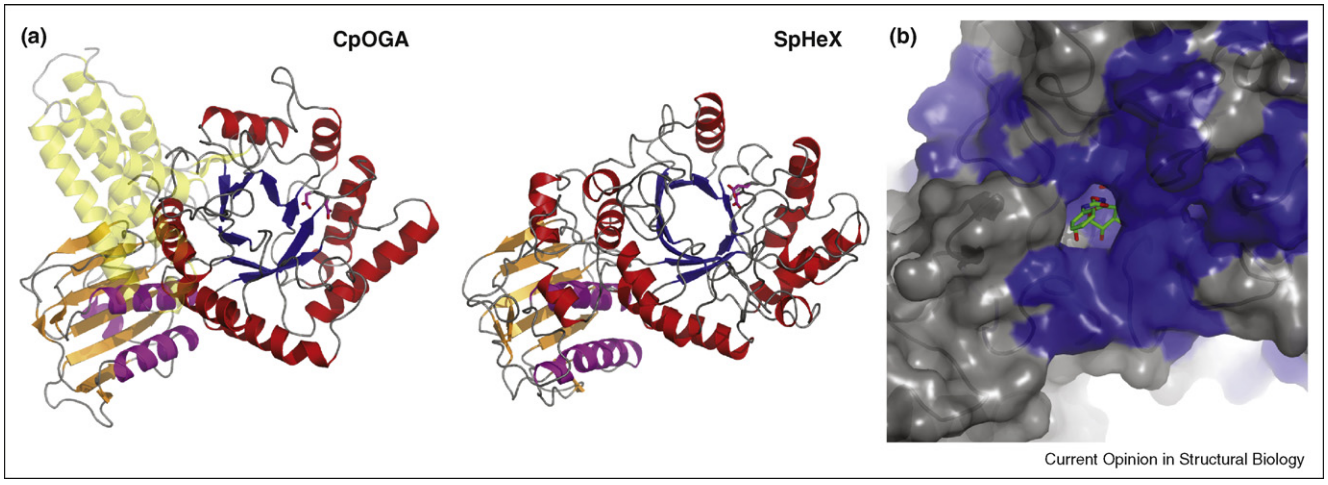
Of particular interest is the 'Yin-Yang hypothesis', proposing that *O*-GlcNAc shows interplay with, and to a certain extent controls levels of, protein phosphorylation [3]. More than 50% of the known *O*-GlcNAc sites have a Pro-Val-Ser motif similar to that recognised by proline-directed kinases [3]; and in some cases *O*-GlcNAc is reciprocal with phosphorylation on some well-studied proteins, such as RNA polymerase II, estrogen receptor and the c-Myc proto-oncogene product. However, other examples of *O*-GlcNAc/phosphorylation interplay have been demonstrated, for instance *O*-GlcNAc can occur adjacent to phosphorylation sites affecting the degree of the phosphorylation, as in p53 [7].

Two enzymes, *O*-GlcNAc transferase (OGT) and *O*-GlcNAc hydrolase (OGA), attach and remove *O*-GlcNAc from proteins, respectively. Until very recently, little was known about the structures and reaction/substrate recognition mechanisms of OGA/OGT. This review covers a recent flurry of studies, covering crystal structures of the bacterial homologues of eukaryotic OGT/OGA and exploitation of these to develop highly potent and selective OGA inhibitors.

O-GlcNAcase — structure by proxy and myths dispelled

The human genome carries three genes encoding enzymes that are able to hydrolyse terminal *N*-acetylglucosamine. These enzymes fall into two distinct glycoside hydrolase families. Human hexosaminidases A and B are members of the CAZY GH20 family [8], and have been extensively studied in terms of their structure, mechanism and inhibition (e.g. [9]). The third enzyme was initially termed hexosaminidase C, but was later discovered to be equivalent to human OGA (hOGA). hOGA is a 92-kDa enzyme formed by two domains: an N-terminal hydrolase catalytic domain (residues 1–700) belonging to the GH84 family [8]

Figure 1



Structure of bacterial OGA. **(a)** Ribbon representations of the *Cp*OGA (PDBID 2CBI [13••]) and *S. plicatus* hexosaminidase (*Sp*Hex, PDBID 1HP5 [41]) structures. The N-terminal α/β domains are shown in orange and purple. The catalytic $(\beta\alpha)_8$ domain is shown in blue and red. The C-terminal domain *Cp*OGA without any apparent sequence homology to hOGA is shown in transparent yellow. The catalytic residues (Asp/Asp for *Cp*OGA, Asp/Glu for *Sp*Hex) are shown as sticks. **(b)** Surface representation of the *Cp*OGA structure (PDBID 2CBJ [13••]) coloured by sequence conservation with hOGA (grey = not conserved, dark-blue = conserved and blue = identical side chains). The PUGNAc inhibitor is shown in a stick representation with green carbon atoms.

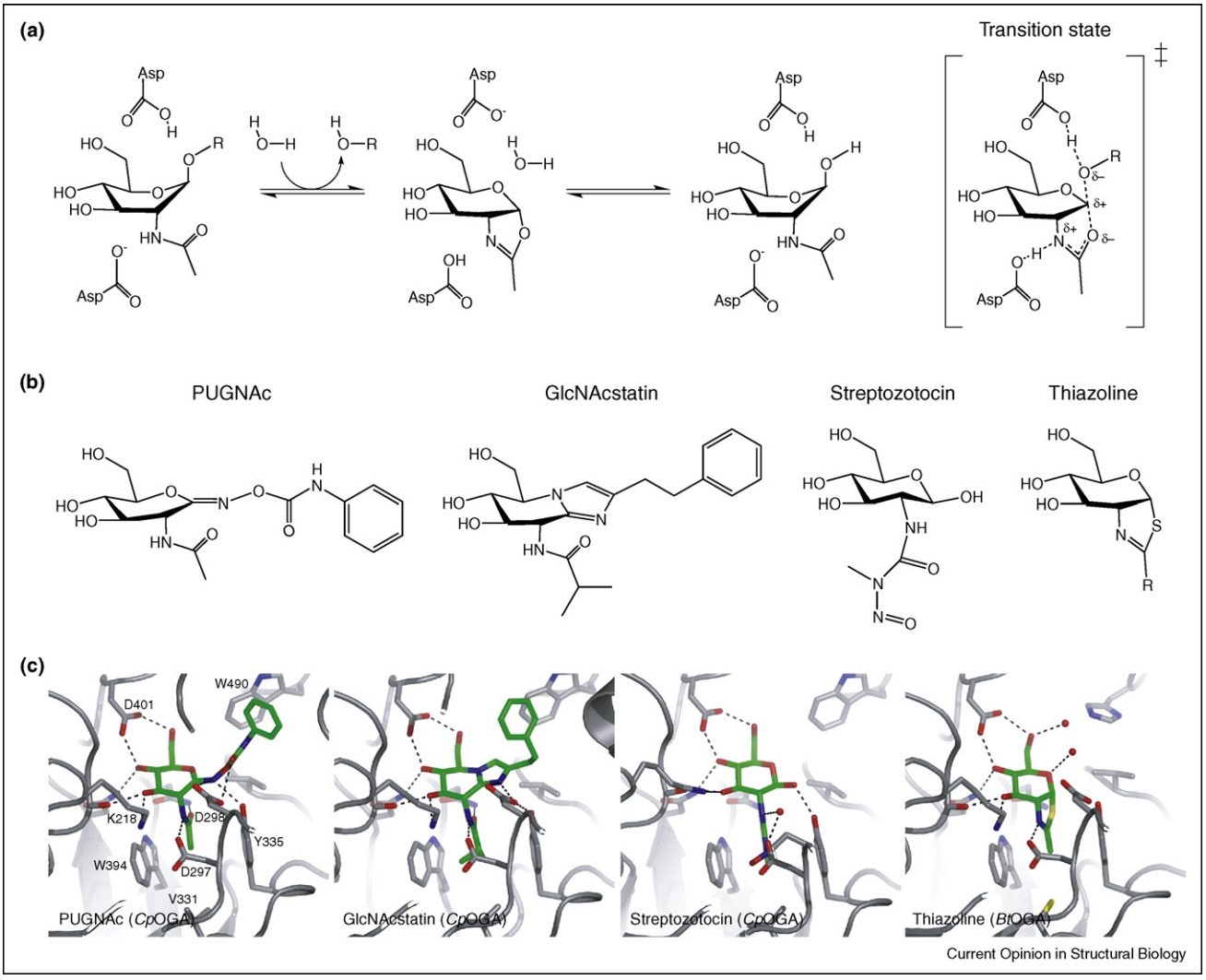
and a C-terminal domain (residues 701–916), which has been proposed to possess histone acetyltransferase activity [10,11]. Until recently no structural data were available for the GH84 family. A first hint that there might be some similarities between the GH20/GH84 enzymes came from an elegant mechanistic study that showed that, like GH20 enzymes, hOGA uses a substrate-assisted catalytic mechanism, proceeding via an oxazolinium ion intermediate formed after the attack of the acetamido group on the anomeric carbon [12••] (Figure 2a). Although hOGA can be expressed and purified yielding samples suitable for biochemical studies, attempts to crystallize the protein have so far failed. Two recent studies noted the presence of several bacterial apparent OGA orthologues in the GH84 family and used these to provide the first insights into OGA structure, through the crystal structures of *C. perfringens* NagJ (*Cp*OGA) and *Bacteroides thetaiotaomicron* hexosaminidase (*Bt*OGA) [13••,14••]. These structures revealed that despite lacking sequence homology, OGA adopts a fold very similar to the GH20 enzymes, including an N-terminal α/β fold and a $(\beta\alpha)_8$ catalytic core (Figure 1a). This also extends to the active site, where two aspartic acids are positioned similarly to a glutamic/aspartic acid pair in the GH20 enzymes, to perform roles in glycoside protonation and assistance of the *N*-acetyl group. Structures of complexes with sugar-derived inhibitors defined the rest of the active site, almost fully conserved with hOGA, revealing an extensive hydrogen bonding network with the sugar hydroxyls and stacking interactions with a tryptophan (Figure 2c). These structures instantly dispelled the myth that these bacterial enzymes were hyaluronidases (they were annotated as such in sequence databases) — the GlcNAc sugar occupies a pocket in the enzyme, leaving no further room for sugars beyond the −1 subsite, later confirmed by biochemical means [15•]. Interestingly, the loop carrying the DD catalytic machinery was shown to undergo large conformational changes upon ligand binding [13••,16]. This is not possible with the GH18, GH20, and GH56 enzymes that carry a DxE motif, as this motif is positioned at the end of β-strand 4 of the $(\beta\alpha)_8$ barrel.

Strikingly, both these bacterial enzymes were shown to possess *O*-GlcNAcase activity towards a broad spectrum of proteins in human cell lysates [13••,14••]. Indeed, the analysis of sequence conservation between *Cp*OGA and hOGA in the context of the *Cp*OGA structure suggests that not only the sugar-binding site is conserved, but also a groove running across the surface of the protein (Figure 1b). While this hints at the possibility of defined interactions between OGA and *O*-GlcNAcylated protein substrates beyond just the OGA–GlcNAc interactions, further experiments are required to test this.

From coordinates to *O*-GlcNAcase inhibitors

For the past decade, two OGA inhibitors, PUGNAc and streptozotocin (STZ), have been extensively used to induce increased levels of cellular *O*-GlcNAc and study the effects on specific cellular processes. Recent structural data obtained from the bacterial OGA homologue *Cp*OGA in complex with these compounds have given insights into their binding modes.

Figure 2



Binding modes of OGA inhibitors. **(a)** Reaction mechanism of *O*-GlcNAc hydrolases using substrate-assisted catalysis. **(b)** Chemical structures of a range of OGA inhibitors. **(c)** The crystallographically determined complexes of bacterial OGAs (ribbon with key side chains shown as sticks) with inhibitors (sticks representation with green carbon atoms) are shown: PUGNAc–*Cp*OGA (2CBJ.pdb) [13••]; GlcNAcstatin–*Cp*OGA (2J62.pdb) [30••]; streptozotocin–*Cp*OGA (2VUR.pdb) [23•]; NAG–thiazoline–*Bt*OGA (2CHN.pdb) [14••].

PUGNAc (Figure 2b) was originally identified as a GH20 hexosaminidase inhibitor, but later also shown to potently inhibit OGA [17]. Recently, insights into the binding mode of PUGNAc to OGA have been obtained through a *Cp*OGA–PUGNAc complex (PUGNAc shows a K_i of 5 nM against *Cp*OGA compared to 52 nM against hOGA) [13••] (Figure 2c). PUGNAc binds, as the expected Z isomer, with the *N*-acetylglucosamine deep into the active site. The phenylcarbamate moiety substituting the O-linkage to a protein substrate is solvent exposed and projects out of the active site onto the surface of *Cp*OGA. The sp^2 C1 carbon induces a ^4E pyranose conformation, similar to the transition state proposed for substrate-assisted catalysis for retaining glycoside hydrolase families GH18, GH20, GH56 and GH84 (Figure 2c). Consistent with this, the PUGNAC 2-acetamido oxygen is positioned within 3 Å from the C1 carbon. Furthermore, PUGNAc is tethered via its hydroxyls by numerous hydrogen bonds to residues that are completely conserved between *Cp*OGA and hOGA (Figures 1 and 2c). The mutation of most of these residues significantly affects PUGNAc binding [13••].

STZ, a *N*-methyl-*N*-nitrosoureido D-glucosamine derivative (Figure 2b), is a natural product that is selectively toxic to pancreatic β cells and is extensively used to induce type I diabetes in animals and to treat cancer of pancreatic islets [18,19]. Cells treated with STZ show elevated *O*-GlcNAc levels, and STZ weakly inhibits OGA, leading some to propose that STZ kills β cells

in an *O*-GlcNAc-dependent manner [20]. Further reports have proposed different inhibition modes, involving either a covalent enzyme–STZ complex [21], or an enzyme-catalysed formation of a bicyclic, oxazoline-bearing tight binding inhibitor [22]. Very recently, a crystal structure of the *Cp*OGA–STZ complex has been reported [23•]. The pyranose ring (in the $_4C^1$ β configuration) occupies a similar position as initially observed for PUGNAc (Figure 2c). While STZ was shown to inhibit *Cp*OGA, no structural or biochemical evidence was found for a covalent interaction of the proposed oxazoline bearing derivative, indeed, the loop carrying most of the catalytic machinery was shown to be disengaged from the inhibitor (Figure 2c). Interestingly, *galacto*-configured STZ, designed on the basis of the complex, shows the expected complete loss of OGA inhibition, yet still induces pancreatic β cell death, providing further evidence against an *O*-GlcNAc-dependent mechanism for STZ-induced cell death.

It rapidly became clear that both PUGNAc and STZ are not suitable tools for studying the cell biology of *O*-GlcNAc. STZ is a weak inhibitor with severe toxicity issues, whereas PUGNAc almost as potently inhibits the lysosomal hexosamidinases as it does OGA. Thus, several studies have been conducted towards the discovery of more potent and selective OGA inhibitors, so far essentially employing two different strategies.

2′-Methyl-α-D-glucopyrano-[2,1-*d*]-δ2′-thiazoline (NAG-thiazoline) had previously been reported as a potent inhibitor of the GH20 family hexosaminidases [24], but was recently discovered to be a low nanomolar OGA inhibitor [12••]. The potency of this compound is derived from its isosterism with the oxazoline reaction intermediate, as demonstrated by the recently obtained crystal structure of the compound in complex with *Bt*OGA [14••] (Figure 2c). The pyranose ring adopts a somewhat distorted $^4C_1$ conformation, with all three hydroxyl-groups (O6, O4 and O3) interacting via hydrogen bonds with conserved residues, identical to the *Cp*OGA–PUGNAC complex. The catalytic residues Asp242 and Asp243 are similarly positioned as the Asp–Asp pair in the PUGNAc–*Cp*OGA structure, compatible with substrate-assisted catalysis. It was then noted that the thiazoline methyl side chain pointed towards an (unoccupied) pocket that is absent in the GH20 enzymes (Figure 2c). Extensive modification of this side chain has led to the identification of NAG-thiazoline derivatives with nM–μM hOGA inhibition that show improved selectivity over the lysosomal hexosaminidases [25,26••].

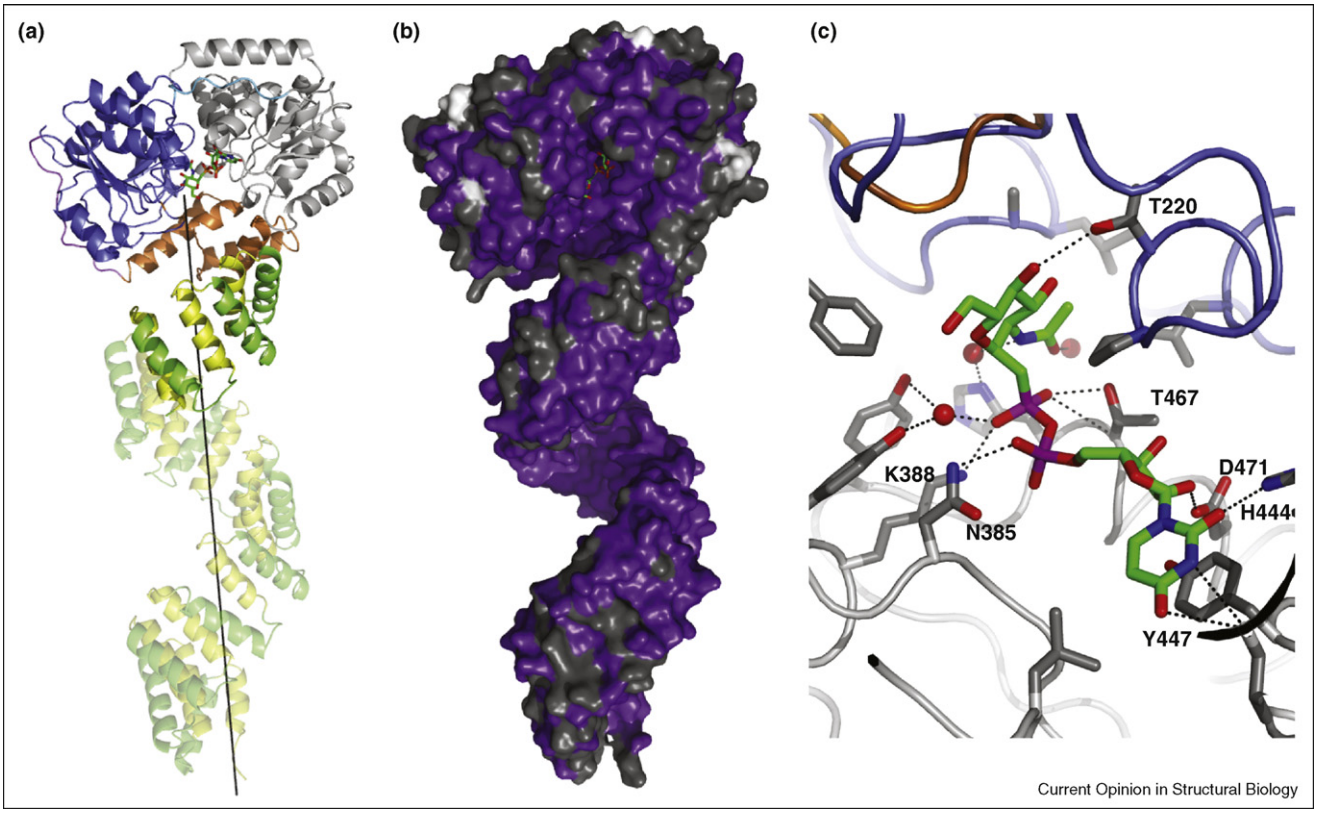
Another approach towards more potent and selective OGA inhibitors has been to expand the glucoimidazoles as exemplified by the parent compound nagstatin, a nanomolar hexosaminidase inhibitor [27]. These compounds are thought to tightly interact with the catalytic machinery not only because of their isosterism with the transition state, but also because of the delocalised positive charge on the imidazole ring [28]. Initial attempts with this scaffold resulted in a PUGNAc–imidazole hybrid, inhibiting OGA in the low μM range, though it was crystallographically demonstrated to have the expected OGA binding mode [29]. Dorfmueller *et al.* exploited the structural data of the *Cp*OGA–PUGNAc complex, and rationally designed GlcNAcstatin ([30••] Figure 2b). This compound competitively inhibits *Cp*OGA with K_i = 4.6 pM and is the most potent GH84 inhibitor reported so far, though its activity against hOGA has not yet been reported. A crystal structure of the GlcNAcstatin–*Cp*OGA complex shows that the sugar moiety of GlcNAcstatin adopts a ^4E conformation, occupies the expected position and the hydroxyl hydrogen-bonds are conserved with the PUGNAc and thiazoline complexes (Figure 2c). The catalytic acid induces protonation of the exo-cyclic imidazole nitrogen as proposed by Vasella *et al.* [28]. This compound achieves 100 000-fold selectivity against the human lysosomal hexosaminidases by the isobutanamido group occupying the extra pocket present in GH84 enzymes (Figure 2c).

OGT — a GT-B fold with a large twist

OGT is expressed as three isoforms, the 110 kDa/78 kDa forms are localised in the nucleus as heterotrimers, and a third of 103 kDa is localised in the mitochondria [3]. The 110 kDa enzyme is composed of two domains separated by a nuclear localisation sequence [31]. The N-terminal domain is composed of multiple tetraticopeptide repeats (TPRs) that are thought to be important for the recognition of large protein substrates [32,33]. The C-terminal domain possesses the glycosyl-transferase activity, belonging to the GT41 family in the CAZY database [34] and using UDP-GlcNAc as the donor substrate.

The first structural data for hOGT were a structure of a number of the N-terminal TPR repeats, that were shown to form a superhelix (Figure 3a), carrying a string of conserved asparagines proposed to contribute to substrate recognition [35•]. Despite works by several labs on the development of expression systems for full-length hOGT, these have so far not yielded crystallization-quality protein samples. In the past few months, two groups have attempted to gain insight into the structure of hOGT by solving the crystal structure of *Xanthomonas campestris* OGT (*Xc*OGT), an apparent bacterial orthologue of the eukaryotic OGTs, in complex with UDP [36••] and a UDP-GlcNAc phosphonate analogue [37••] (Figure 3). Given the significant level of sequence conservation (36%) and the presence of several TPRs, these studies used the *Xc*OGT structure as a guide for probing the molecular mechanisms of the human enzyme. *Xc*OGT, and thus hOGT, possesses the typical bilobal GT-B fold domain and three complete TPRs followed by two pairs of antiparallel helices, forming two TPR-like

Figure 3



Structure of *Xc*OGT. **(a)** Ribbon representation of the *Xc*OGT structure (PDBID 2VSY [37••]), showing the two halves of the GT-B fold (grey/blue), the TPRs (green/yellow, the superimposed structure of the hOGA TPRs is also shown (transparent)) and the TLRs (orange). The superhelical axis is shown as a black line. The UDP-GlcNAc analogue is shown as a stick representation with green carbon atoms. **(b)** Surface representation of the *Xc*OGT structure and the superimposed structure of the hOGA TPRs, coloured by sequence conservation between metazoan OGTs (white (non-conserved residues) via grey (conserved residues) to purple (identical residues)). The UDP-GlcNAc analogue is shown as a stick representation with green carbon atoms. **(c)** Details of the *Xc*OGT active site (ribbon (colours as in panel A), with side chains shown as sticks with grey carbons) and interactions with the UDP-GlcNAc analogue (stick representation with green carbon atoms). The conformation of the active site lid loop in the apo-*Xc*OGT structure is shown in orange. Where hydrogen bonds are made to backbone atoms, these are shown to the C_α atom instead. Water molecules interacting with the ligand are shown as red spheres.

repeats (TLRs) (Figure 3a). As with other GT-B enzymes, the active site is positioned between the two halves of the GT-B fold (Figure 3a). The most striking aspect of the structure is perhaps that the TPRs/TLRs are facing/closely associated with the active site of (*Xc*OGT), forming an elongated, continuous and evolutionary conserved groove from the donor binding site to the end of the TPR superhelix, providing a structural explanation for the participation of the TPRs in recognition of protein/peptide substrates (Figure 3). This is quite different from most other TPR proteins, where they are typically attached via a flexible linker [38]. Interestingly, point mutations on this surface near the active site affected hOGT activity towards larger protein substrates [37••].

The structures of the *Xc*OGT complexes with UDP/UDP-GlcNAc phosphonate analogue revealed that UDP is tethered by hydrophobic interactions with a tyrosine, hydrogen bonds to the protein from the uridine/ribose and several basic residues near the pyrophosphate (Figure 3c). The sugar is inserted between three loops, one of them burying the *N*-acetyl group while leaving the β face of the anomeric carbon accessible. One of these loops, termed the active site lid, assumes different conformations in the *Xc*OGT structures and is the site of inactivating mutants of *spy*, a plant OGT orthologue ([39], Figure 3c). Although probing of the hOGT active site by mutagenesis yielded several inactive mutants, the precise catalytic mechanism of OGT remains to be established.

O-GlcNAc structural biology — what we do not know yet

Although our understanding of the mechanisms involved in *O*-GlcNAcylation has dramatically increased over the past few years, many important questions remain unanswered. For OGA, two key issues remain unsolved. While we now understand how the enzyme interacts with sugars

and catalyses the hydrolytic step, the mechanism through which it interacts with the peptide backbone remains unknown. While with micromolar concentrations of the enzyme all *O*-GlcNAc on proteins in human cell lysates can be removed, distinct patterns of sequence conservation are apparent in a groove beyond the sugar-binding site. An OGA–peptide complex would help us understand the possible mechanisms of recognition and specificity. Furthermore, OGA does not just consist of a glycosidase catalytic core. A domain with very distant homology to histone acetyl transferases resides at the OGA C-terminus and the function of this domain is completely unknown. A structure of this domain may lead to a testable biochemical hypothesis. Finally we will need to understand the presence of genes coding for proteins with OGA/OGT activity in bacterial genomes — will these provide examples of bacterial *O*-GlcNAc or are they perhaps involved in virulence?

It is clear that a huge amount of effort has gone into the development of increasingly potent and specific OGA inhibitors, leading to the generation of useful tools for the cell biologist. However, perhaps more interesting than driving up general *O*-GlcNAc levels with an OGA inhibitor would be the chemical reduction of *O*-GlcNAcylation with an OGT inhibitor. While the recent OGT structures will provide a framework for the rational development/evaluation of inhibitors, this is as yet an almost completely unexplored area. Although pilot OGT HTS studies have been published (e.g. [40]), leading to the identification of inhibitors in the micromolar range, the mode of inhibition/binding mode of these is currently unknown.

While specific sequons exist for *N*-glycosylation, leading to fairly accurate prediction of *N*-glycosylation sites from sequence alone, this is not the case for *O*-GlcNAcylation. Although proteomic studies have identified hundreds of *O*-GlcNAcylated proteins, the precise site of modification is known for only a few [3]. Given the fact that *O*-GlcNAc transfer is, as far as currently known, carried out by a single gene product, clues to selectivity towards only a subset of cytosolically available serines/threonines must lie in the OGT structure. The challenge here is to determine complexes with short peptides or entire substrate proteins to unravel the mechanisms of substrate recognition and specificity.

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