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Stereochemical divergence of polyprenol phosphate glycosyltransferases

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Abstract

In the three domains of life, lipid-linked glycans contribute to various cellular processes, ranging from protein glycosylation to glycosylphosphatidylinositol anchor biosynthesis to peptidoglycan assembly. In generating many of these glycoconjugates, phosphorylated polyprenol-based lipids are charged with single sugars by polyprenol phosphate glycosyltransferases. The resultant substrates serve as glycosyltransferase donors, complementing the more common nucleoside diphosphate sugars. It had been accepted that these polyprenol phosphate glycosyltransferases acted similarly, given their considerable sequence homology. Recent findings, however, suggest that matters may not be so simple. In this Opinion, we propose that the stereochemistry of sugar addition by polyprenol phosphate glycosyltransferases is not conserved across evolution, even though the GT-A fold that characterizes such enzymes is omnipresent.

Keywords

Dolichol phosphate; dolichol phosphate glucose synthase; dolichol phosphate mannose synthase; polyprenol phosphate; protein glycosylation; stereochemistry

Glycosylation of linear, long-chain polyprenol phosphates across evolution

In addition to serving as key constituents of the basic building blocks of biological membranes, as currency for energy storage in the form of triacylglycerides and as vital intracellular signaling molecules (for recent reviews, see [1–3]), lipids also serve as platforms upon which diverse glycoconjugates are assembled [4,5]. In many cases, glycoconjugate biosynthesis begins with the addition of a single carbohydrate to a phosphorylated, membrane-associated polyprenol-based lipid carrier by the actions of either **polyprenol phosphate phosphoglycosyltransferases** (PGTs) (see Glossary) [6] or **polyprenol phosphate glycosyltransferases** (GTs) [7]. Such enzymes are important for the

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synthesis of substrates for N-glycosylation, a post-translational modification in which select asparagine residues in target proteins are modified by glycans originally assembled on a phosphorylated polyprenol carrier [8–10]. The membrane-resident carriers are the uniformly unsaturated **polyprenol** phosphates in Bacteria and the **dolichol** monophosphates and/or dolichol diphosphates in Eukarya and Archaea [5,11–13] (Figure 1).

As part of the endoplasmic reticulum (ER)-localized phase of N-linked protein glycosylation in yeast and most higher eukaryotes, dolichol monophosphate (C_{70–110} [5,11,12]) is charged with either glucose (Glc) or mannose (Man) in reactions catalyzed by the polyprenol phosphate GTs, dolichol phosphate glucose (DPG) synthase (e.g. Alg5 [14]) or dolichol phosphate mannose (DPM) synthase (e.g. Dpm1 [15]), using UDP-Glc or GDP-Man as substrates, respectively [16–18]. Once activated as the corresponding dolichol monophosphate derivatives, Glc and Man are subsequently transferred to the non-reducing end of a dolichol diphosphate-linked heptasaccharide intermediate to afford the tetradecasaccharide (N-acetylglucosamine (GlcNAc)₂Man₉Glc₃) that is transferred to selected asparagine residues in target proteins at the gateway to the secretory pathway [8]. Thus, the dolichol phosphosugars serve as alternative donors that are implemented when the acceptor substrates are membrane-associated in cellular compartments where the corresponding nucleoside diphosphate (NDP)-sugars are not available.

In Bacteria, where N-glycosylation seems to be limited to delta and epsilon proteobacteria [9], N-linked glycans are assembled onto a C₅₅ polyprenol (undecaprenol) diphosphate carrier. In contrast, bacterial O-mannosylation, in which a glycan is linked via a Man to selected serine or threonine residues in the target protein, begins with sugar transfer from an GDP-Man donor to a polyprenol monophosphate carrier [19]. The enzyme responsible, a polyprenyl monophosphomannose synthase, shows significant sequence homology to eukaryal Dpm1 [20]. Subsequently, the polyprenol monophosphate-bound Man is delivered to the target protein by a protein O-mannosyltransferase [21]. In addition, some bacteria recruit polyprenol phosphates charged with single sugars in the biosynthesis of lipid A, a major component of the lipopolysaccharide that comprises the outermost layer of Gram-negative species [22]. In polymyxin-resistant mutants of *Escherichia coli* and *Salmonella typhimurium*, ArnC transfers the sugar moiety from UDP-4-deoxy-4-formamido-L-arabinose (L-AraFN) to undecaprenol phosphate [23], while in *Francisella tularensis*, the causative agent of tularemia, FlmF1 and FlmF2 respectively transfer Glc and galactosamine (GalN) from UDP-Glc and UDP-GalN to the same lipid [24].

In Archaea, N-glycosylation is an almost universal post-translational protein modification that culminates in an extremely diverse set of protein-bound glycans [25–27]. Although current understanding of archaeal N-glycosylation is limited, it is known that both dolichol monophosphate and dolichol diphosphate serve as the lipid carriers upon which glycans for N-linked protein glycosylation are assembled [13]. Indeed, in Archaea, sugar-charged dolichol monophosphates serve as both sugar donors and as carriers for further sugar addition during N-linked glycan assembly [28,29]. Moreover, DPG and DPM synthases, as well as other polyprenol phosphate GTs, involved in N-glycosylation have been identified in several species. In the halophile *Haloferax volcanii*, AglJ is a DPG synthase [30,31] and AglD is a DPM synthase [28,32], while in the methanogen *Methanococcus voltae*, AglK

transfers a GlcNAc to dolichol monophosphate in the initial step of N-linked protein glycosylation [29]. Additionally, *Hfx. volcanii* Agl6 is a polyprenol phosphate GT believed to add a hexose to dolichol monophosphate during assembly of an N-linked tetrasaccharide generated in conditions of decreased salinity [33], while in *Halobacterium salinarum*, evidence supports the assignment of VNG1053G as a DPG synthase [34,35]. Additional polyprenol phosphate GTs have also been described, although their roles in the cell have yet to be biochemically defined. For example, PF_0058 is a DPM synthase, which has recently been structurally characterized by X-ray crystallography [36], from the hyperthermophile *Pyrococcus furiosus*. However, as an N-acetylgalactosamine (GalNAc), which is part of the N-linked *P. furiosus* heptasaccharide, has been shown to be directly linked to both dolichol monophosphate and asparagines in glycoproteins in this organism [37–39], the contribution of PF_0058 to N-glycosylation remains unclear. Although the N-glycosylation pathway in *P. furiosus* has yet to be delineated, dolichol monophosphate-bound Man could potentially contribute either or both mannoses found as part of the N-linked heptasaccharide in this species [37]. Finally, *Hfx. volcanii* HVO_1613 modifies dolichol monophosphate with a hexose distinct from Glc or Man but apparently does not contribute to N-glycosylation [30].

Given the sequence similarities amongst polyprenol phosphate GTs identified across domains of life, it had been assumed that all of these enzymes employ a similar catalytic mechanism. This, however, appears to be an over-simplification.

Different mechanisms for different polyprenol phosphate GTs?

It had been generally accepted that polyprenol phosphate GTs, assigned to the abundant **CAZy** (Carbohydrate-Active EnZyme; <http://www.cazy.org/Welcome-to-the-Carbohydrate-Active.html>) GT2 glycosyltransferase family [40], may act similarly, given their considerable structure and sequence homology. Indeed, examination of an archaeal DPM synthase from *P. furiosus* [36] and GtrB, a bacterial DPG synthase from *Synechocystis* sp. PCC6803 that participates in O-antigen biosynthesis [41], the only two polyprenol phosphate GTs for which structural information is available, reveals that their GT domains assume the prototypic **GT-A fold** predicted for inverting GT2 family members [40]. The stereochemical course of polyprenol phosphate GT-catalyzed reactions, exemplified by eukaryal Agl5 and Dpm1, supports a direct displacement via an S_N2 -like mechanism involving general base catalysis that increases the nucleophilicity of the attacking group [7] (Figure 2A). These reactions proceed with inversion of stereochemistry at the **anomeric center**; the NDP-sugar substrate is α -linked, whereas in the dolichol monophosphate product it is β -linked.

Based on the sequence similarities of archaeal polyprenol phosphate GTs to their eukaryal counterparts involved in the later steps of N-linked protein glycosylation, it was assumed that in Archaea, the monosaccharides added to dolichol monophosphate would also feature the β -anomeric configuration. Unexpectedly, however, dolichol monophosphate-linked monosaccharides that appear at the inception of archaeal N-glycosylation pathways are instead found as the α -anomers. This was confirmed by analysis of the configuration of the dolichol monophosphate-GlcNAc formed by *M. voltae* AglK using ^{31}P -decoupled ^1H NMR and analysis that determined the $J_{1,2}$ coupling constant [29]. Diagnostic regions of the NMR

spectrum are illustrated in Figure 3, alongside that of β -dolichol monophosphate glucose for comparison.

Similar observations have been made with *Hfx. volcanii*. When *Hfx. volcanii* cells are grown in medium containing 1.25–1.75 M NaCl, conditions considered as low salinity for this organism [42], dolichol monophosphate is modified by a tetrasaccharide linked via a sulfated hexose, presumably galactose (Gal), in a reaction catalyzed by Agl6 [33,43]. Here too, ^1H NMR analysis confirmed that the dolichol monophosphate-linked hexose showed α -anomeric stereochemistry. These findings suggest that in Archaea, at least in some cases, polyprenol phosphate GTs invoke a **retaining mechanism**. The catalytic mechanism of retaining GTs is less well defined, with several different mechanisms, including a double displacement mechanism or a front-face mechanism involving either a concerted or stepwise reaction, proposed [44] (Figure 2B). Indeed, although the NDP-sugar binding sites appear to be quite conserved, an examination of the details of the acceptor substrate binding sites of the solved structures of retaining GT-A fold-containing GTs does not reveal common principles. This latter observation may in part be due to the highly varied nature of the acceptor substrates [7].

Why does polyprenol phosphosugar stereochemistry matter?

Across evolution, the transfer of lipid-linked glycans to target asparagine residues is mediated by an oligosaccharyltransferase. Bacterial PglB and archaeal AglB, each acting alone, and eukaryal Stt3, acting alone in lower eukaryotes or as part of a heterooligomeric complex in higher organisms, are homologous proteins that catalyze the reaction [25,45,46]. These enzymes rely on an **inverting mechanism** whereby an α -linked glycan in the lipid diphosphate-linked substrate is processed to yield the asparagine amide β -linked glycan in the glycoprotein product. In the eukaryal and bacterial systems, where the N-linked glycan is delivered from either dolichol diphosphate or undecaprenol diphosphate, respectively, the α -anomeric stereochemistry of the lipid diphosphate-linked sugar is maintained in the first membrane-committed step. For example, addition of the first sugar of the bacterial N-linked glycan in *Campylobacter jejuni* is carried out by PglC, a PGT which catalyzes the transfer of 2,4-diacetamidobacillosamine (diNAcBac) phosphate from the corresponding UDP-diNAcBac to undecaprenol monophosphate [47]. Therefore, the enzyme attacks at the β -phosphate group of the UDP-sugar, preserving the stereochemistry at the anomeric center. The same is true in Eukarya, where UDP-GlcNAc provides the phosphosugar for transfer to dolichol monophosphate in a reaction catalyzed by the GlcNAc-phosphate transferase Alg7. The same reaction also occurs in some Archaea (Figure 4 (Key Figure), top reaction). In contrast, the polyprenol phosphate GTs that initiate the assembly of dolichol monophosphate-bound glycans in Archaea must retain the α -anomeric configuration of the linking sugar, delivered from the UDP-sugar, since these enzymes attack directly at the anomeric center (Figure 4, middle reaction). In the case of Alg5 and Dpm1, the DPG and DPM synthases that respectively add Glc and Man to dolichol monophosphate (from where these sugars are transferred to the dolichol diphosphate-linked heptasaccharide intermediate in the eukaryal dolichol pathway [18]), the stereochemistry of the added saccharides does not impact the later oligosaccharyltransferase-catalyzed reaction (Figure 4, bottom reaction).

Concluding remarks and future perspectives

If eukaryal and archaeal polyprenol phosphate GTs indeed catalyze similar reactions yet with different stereochemistry, then one would imagine that the two versions of the enzyme can be distinguished at the sequence and/or structural levels. As preliminary comparisons of the sequences and membrane topologies of those archaeal enzymes known to be involved in N-glycosylation with their eukaryal counterparts fails to reveal obvious distinction, the basis for the apparent reliance on mechanisms involving distinct stereochemistry remains unclear (see Outstanding Questions). The availability of structural information on both inverting and retaining polyprenol phosphate GTs will likely prove central to such efforts.

Outstanding Questions Box

- What is the evolutionary history of polyprenol phosphate glycosyltransferases?
- Why are dolichol or polyprenol monophosphates used as glycan lipid carriers in some cases and dolichol or polyprenol diphosphates used in other instances?
- Are there additional roles for polyprenol or dolichol mono- and/or diphosphate-linked glycans that remain to be described?
- Why are polyprenol monophosphate sugars used as glycan donors in some instances and nucleoside phosphate sugars in others?
- Do all archaeal polyprenol phosphate glycosyltransferases employ a retaining mechanism? Do any of their eukaryal or bacterial counterparts?
- What is the catalytic mechanism employed by retaining glycosyltransferases? Can this be predicted from structure?
- Will the availability of additional structural information on retaining and inverting polyprenol phosphate glycosyltransferases make it possible to predict the stereochemical outcome of the reactions from structure alone?

Finally, if the archaeal N-glycosylation process represents a precursor to the more elaborate eukaryal system, the finding that the two domains invoke lipid-linked glycan donors with distinct anomeric stereochemistry carries implications for the evolution of N-glycosylation. One can ask why eukaryal Alg5 and Dpm1 failed to maintain the stereochemistry of their archaeal counterparts. It is also unclear why the later steps of the N-linked glycosylation pathways of higher eukaryotes employ Glc and Man bound to dolichol monophosphate rather than simply extending the dolichol diphosphate-bound heptasaccharide that forms the core of their N-linked glycans using NDP-sugar donors. Certainly, in part this is due to the absence of the corresponding NDP-sugar donors in the ER. While it is conceivable that the dolichol diphosphate-linked tetradecasaccharide transferred to nascent proteins could be fully assembled using available NDP-sugars in the cytoplasm, and not just the heptasaccharide core, it may be difficult to translocate such an elaborate glycan across the ER membrane. Indeed, across Eukarya, the longest dolichol diphosphate-linked

polysaccharide translocated across the ER membrane contains seven sugars [13, 48]; the same is true in the bacterial N-glycosylation system exemplified by *C. jejuni* [9, 46, 49]. At the same time, it is noteworthy that the protist *Trichomonas vaginalis*, a lower eukaryote, encodes Alg5 and Dpm1 homologues, yet N-linked glycans in this organism are not elaborated with Glc and/or Man derived from the corresponding dolichol monophosphate carriers [50].

N-glycosylation is a post-translational modification that expands the scope of biological interactions and communication, given the enormous diversity possible in terms of the size, content and architecture of the N-linked glycans that decorate modified proteins. As such, it is understandable why this protein-processing event is so ubiquitous across evolution. It is, however, less clear why nature presents so many different variations on a common theme, including the apparently domain-specific use of stereochemically-distinct polyprenol phosphate GTs considered here. Continued investigation into N-glycosylation across the living world will provide insight into this and other questions, and likely reveal new twists on what was considered a simple story.

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Glossary

Anomeric center

In the cyclized pyranose or furanose forms, monosaccharides feature an asymmetric center created by formation of an intramolecular acetal (or ketal) between a sugar hydroxyl group and the aldehyde (or ketone) group. Two stereoisomers called anomers are formed because the anomeric hydroxyl group can assume two possible configurations. When the configurations are the same at the anomeric carbon and the stereogenic center furthest from the anomeric carbon, the monosaccharide is defined as the α -anomer. When the configurations are different, the monosaccharide is defined as the β -anomer. This implies that for the common D-hexoses/hexosamines, the C5-stereochemistry defines the anomeric designation and so the α -anomeric substituent (e.g. UDP or DolP) would be axial and the β -anomeric substituent would be equatorial.

CAZy

The CAZy database is a curated site documenting information on structurally-related catalytic and carbohydrate-binding modules (or functional domains) of enzymes that degrade, synthesize or modify glycans.

Dolichol

Dolichols are polyprenoid alcohols comprising linearly linked isoprene subunits, with a hydroxyl group in the saturated α -isoprene subunit at the terminus of the polyprenol. In Archaea, dolichols also present a saturated ω -isoprene and in some cases, additional saturation at the internal isoprene units. The α -isoprene is generally of the (S) configuration.

GT-A fold

Glycosyl transferases with a GT-A fold include two closely abutting $\beta/\alpha/\beta$ Rossmann-like domains. GT-A enzymes are generally dependent on a divalent metal ion, typically Mg(II) or Mn(II), for activity. The metal ion is coordinated by a relatively well-conserved DXD motif within the glycosyltransferase active site and assists in binding to the nucleotide sugar donor and facilitating nucleoside diphosphosphate departure. GT-A-fold GTases can support a retaining or inverting mechanism.

Inverting mechanism

A reaction mechanism which affords a product with the opposite configuration at the anomeric center to the substrate.

Polyprenol

Polyprenols are polyisoprenoid alcohols comprising linearly-linked isoprene subunits with a hydroxyl group at the α -terminus.

Polyprenol phosphate glycosyltransferases

Enzymes that act to transfer a monosaccharide from an NDP-sugar to a polyprenol phosphate acceptor to create a new polyprenol phosphosugar.

Polyprenol phosphate phosphoglycosyltransferases

Enzymes that transfer a C1'-phosphosugar from an NDP-sugar to a polyprenol phosphate acceptor to create a new polyprenol diphosphosugar.

Retaining mechanism

A reaction mechanism which affords a product with the same configuration at the anomeric center as the substrate.

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Trends Box

- Despite their considerable sequence and structural homology, polyprenol phosphate glycosyltransferases can act with different reaction mechanisms and stereochemical outcomes
- In N-linked protein glycosylation, sugars added to dolichol monophosphate carriers present either the α - or the β -configuration
- Novel roles for glycosylated phosphoprenol carriers other than contributing to N-glycosylation have been described

Eukarya

Archaea

Bacteria

Figure 1.

Structures of the fully unsaturated polyprenol and dolichol phosphates implicated in glycan assembly. The polyprenol length, the number of *Z* and *E* isoprene units (*m* and *n*, respectively), and the degree of isoprene unsaturation are species-dependent. Eukarya and Archaea feature dolichols, which are distinguished from the polyprenols used in Bacteria by a saturated α -isoprene unit. In most Eukarya (top panel), $n = 2$ and $m = 12\text{--}17$ ($C_{75}\text{--}C_{100}$), with the exception of plants, where $n = 3$ and $m = 6\text{--}37$ [11,12]. Archaea (middle panel) contain dolichols of varying lengths ($C_{30}\text{--}C_{70}$; $n = 2\text{--}4$) and degrees of unsaturation (indicated by the dashed bond) beyond a saturated α - and ω -isoprene unit [13]. Most Bacteria (lower panel) use fully unsaturated polyprenols ($n = 2$ and $m = 7\text{--}9$). Undecaprenol,

the C₅₅ unsaturated polyprenol ($n = 2$, $m = 8$), is used in bacterial N-glycosylation [5]. The positions of the α - and ω -isoprene units are indicated on the eukaryal lipid.

Figure 2.

The catalytic mechanisms of inverting and retaining polyprenol phosphate GTs. A. The inverting polyprenol phosphate GTs that share a common GT-A fold are generally believed to adopt an S_N2 , direct-displacement mechanism wherein the UDP leaving group is coordinated via Mg(II) to an Asp-Xaa-Asp motif that is generally well-conserved amongst all the GT-A fold GTases. The polyprenol phosphate nucleophile is proposed to be in the dianionic form, without the need for a discrete general base, but stabilized by a local arginine-rich environment [41]. B. The mechanism of the retaining enzymes similarly would involve UDP-sugar binding but in this case with partial or complete departure of the UDP leaving group prior to attack by polyprenol phosphate. Structures of different GT-A fold retaining GTases are known [7], however, there is not a commonly held mechanism because of the diversity of glycosyl acceptors with different reactivity and the limited number of known structures. In each panel, the anomeric center is highlighted in yellow. Pren, polyprenol. The double cross indicates the transition state intermediate.

Figure 3.

NMR analysis of dolichol monophosphate sugar products generated by *M. voltae* AglK and *S. cerevisiae* Alg5. The products of AglK- and Alg5-mediated dolichol monophosphate glycosylated are shown at top of the left and right panels, respectively. Below, the ^{31}P coupled and decoupled spectra of each product are shown. Spectra acquired without ^{31}P decoupling are complicated due to long range ^{31}P coupling (red: H-C(1)-O-P). Therefore, ^{31}P decoupling is performed to disambiguate and highlight the H-C(1)-C(2)-H coupling constant. The predicted coupling constants between the anomeric proton ($\text{C}_1\text{-H}$) and $\text{C}_2\text{-H}$ are 3–4 Hz for the α -linkage and 6–8 Hz for the β -linkage. The values observed in each case are in good agreement with these predictions.

Figure 4.

The stereochemistry of sugar addition to phosphorylated polyprenols involved in N-glycosylation. Polyprenol phosphate PGTs catalyze synthesis of α-linked polyprenol diphosphate-linked sugars, which serve as carriers for N-glycan assembly in Eukarya, delta and epsilon proteobacteria and some Archaea (top reaction). Retaining polyprenol phosphate GTs mediate synthesis of α-linked polyprenol phosphate-linked sugars, which serve as carriers for N-glycan assembly in Archaea (middle reaction). Inverting polyprenol phosphate GTs catalyze the synthesis of β-linked polyprenol phosphate-linked sugars as alternative glycosyl donors to NDP-activated sugars (bottom reaction). Dol, dolichol; GT, polyprenol phosphate glycosyltransferase; Nb, nucleobase; PGT, polyprenol phosphate phosphoglycosyltransferase; Pren, polyprenol. The sugar drawn in blue corresponds to a hexose or a hexosamine.