Polysaccharide co-polymerases: the enigmatic conductors of the O-antigen assembly orchestra

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Received July 15, 2012; revised August 27, 2012; accepted August 30, 2012

Edited by Andrew H. Wang

The O-antigen lipopolysaccharides on bacterial surface contain variable number of oligosaccharide repeat units with their length having a modal distribution specific to the bacterial strain. The polysaccharide length distribution is controlled by the proteins called polysaccharide co-polymerases (PCPs), which are embedded in the inner membrane in Gram-negative bacteria and form homo oligomers. The 3D structures of periplasmic domains of several PCPs have been determined and provided the first insights into the possible mechanism of polysaccharide length determination mechanism. Here we review the current knowledge of structure and function of these polysaccharide length regulators.

Keywords: crystal structure/LPS synthesis/O-antigens/polysaccharides

Introduction

The outer membrane of Gram-negative bacteria provides a protective shield guarding bacteria against environmental and chemical insults. Its outer leaflet is almost entirely composed of the glycolipid constituent known as the lipopolysaccharide (LPS), which consists of three chemically distinct segments: the lipid A, a core oligosaccharide and in some bacteria a carbohydrate polymer known as the O-antigen. While the carbohydrate portion of the inner core tends to be highly conserved across bacterial species, the O-antigen exhibits remarkable diversity giving rise to a large number of bacterial polysaccharide polymers making up the capsule as well as for the enterobacteria common antigen biosynthesis (Valvano, 2003; Whitfield, 2006). In the Wzy-dependent pathway, the biosynthesis of the O-antigen commences on the cytosolic side of the inner membrane where the individual O-units are assembled on the undecaprenyl diphosphate lipid carrier by the sequential action of several glycosyltransferases (Valvano, 2003). The O-units bound to the lipid carrier are transported by a dedicated transporter known as the Wzx flippase across the membrane and enter the periplasm (Marolda et al., 2006b). Once in the periplasm, the O-units become the substrates for the O-antigen Wzy polymerase, which transfers the growing chain to the non-reducing end of the new O-unit forming a glycosidic bond (Robbins et al., 1967). The chain extension terminates with the O-antigen is transferred from the undecaprenyl diphosphate to the oligosaccharide core of the Lipid A molecule by the WaaL ligase while the lipid carrier is recycled to its monophosphate form (El Ghachi et al., 2004; El Ghachi et al., 2005; Tatar et al., 2007).

Length regulation of O-antigen polysaccharide chain

In most bacteria, the mature O-antigen polysaccharide shows a narrow length distribution. This can be readily seen when the O-antigen molecules are subjected to the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis (Fig. 1) (Marolda et al., 2006a). The gene responsible for the control of the O-antigen length was first identified by Batchelor et al. (1991) and was named rol for regulator of O-antigen length or cld for chain length determinant (Bastin et al., 1993) and is now referred to as WzzB. The gene encodes a 36-kDa protein containing two transmembrane domains and a large periplasmic domain (Batchelor et al., 1991). Morona and colleagues (Murray et al., 2003) identified a second O-antigen chain length regulator protein in Salmonella typhimurium responsible for the biosynthesis of a very long O-antigen containing approximately 100 repeat units (Murray et al., 2003). This protein is encoded by fepE.
Those involved in the O-antigen processing in enteric bacteria belong to the PCP-1 class and consist of a large periplasmic domain flanked by two transmembrane helices (Valvano, 2003; Tocilj et al., 2008). Proteins involved in the capsular polysaccharide export and containing a C-terminal cytosolic tyrosine autokinase domain belong to PCP-2A class, while PCP-2B proteins mediate the capsule export in Gram-positive bacteria and are phosphorylated by a kinase encoded on a separate gene (Lee et al., 2008; Olivares-Illana et al., 2008). PCP-3 proteins (exemplified by E.coli KpsE) do not appear to contain the C-terminal transmembrane domain but instead are thought to associate with the inner membrane at the C-terminus (Phoenix et al., 2001).

**PCPs structure**

Cross-linking studies revealed that PCPs implicated in the O-antigen assembly form homo-oligomeric complexes. Such experiments showed the presence of higher molecular weight bands on SDS-PAGE gels of the cross-linked PCPs (Daniels and Morona, 1999). Nevertheless, the precise stoichiometric constitution of the oligomeric chain length regulators in vivo remained unresolved. The first 3D structural information on the periplasmic domains of the PCP demonstrated their tendency to form larger oligomers of varying size (Tocilj et al., 2008). These studies provided structural information for the periplasmic domains of three PCPs, the WzzB from S.typhimurium, FepE from E.coli and WzzE also from E.coli. Although their amino acid sequences show very limited similarity with the pairwise identity being ~23%, the structures of the protomers of the three PCPs exhibited the same 3D fold and were highly similar in terms of the main chain confirmations (Tocilj et al., 2008). Each protomer consists of a base domain containing a four-stranded antiparallel β-sheet flanked by several helices. One of these helices is ~100 Å long (referred to as the central helix) and is followed by two helices returning the chain toward the base domain (Fig. 2). The N- and C-termini of the protomer are close to each other at the bottom of the molecule where the transmembrane helices would be located in the full-length protein. Different PCPs assembled into different oligomers in the crystals. WzzB, FepE and WzzE were found in pentameric, nonameric and the octameric arrangements, respectively (Fig. 3). The protomers in these oligomers interact with each other side-by-side along the entire length of the molecule, although the residues involved in these contacts differ between the PCP protomers (Tocilj et al., 2008). The only exception is the initial structure of WzzBST, which is a pentamer in which the inter-subunit interactions are formed between the base domains only. Such loose associations among the protomers within the oligomer place a question mark on the physiological relevance of this oligomeric organization.

**Polysaccharide co-polymerases**

**Polysaccharide co-polymerases classification**

The LPS O-antigen chain length regulator proteins are part of a larger bacterial protein superfamily known as the polysaccharide co-polymerases or PCPs (Morona et al., 2000; Tocilj et al., 2008; Cuthbertson et al., 2010). This superfamily includes the proteins implicated in the export of complex polysaccharides in both Gram-negative and Gram-positive species. They are subdivided into several different classes depending on the additional structural features in the proteins.
control while noting the importance of the C-terminus for the specification of a given O-antigen chain length. These observations were also corroborated by findings of Franco et al. (1998) who reported that small differences in the O-antigen length distribution imparted by the closely related chain length regulators from several *E.coli* serotypes. Papadopoulos and Morona (2010) used a very different approach employing the non-specific insertion of 6-amino acid

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**Fig. 2.** Crystal structures of the protomers of WzzB, FepE and WzzE shown from two sides.

**Fig. 3.** The oligomeric states of the periplasmic domains of various PCP-1 class proteins revealed by X-ray crystallography.
linkers throughout the polypeptide chain to establish functionally important motifs.

Chimeric PCPs

With the knowledge of the 3D structures of some PCPs and the architecture of the oligomers, the question of which region(s) of the PCPs actively participate in polysaccharide length determination could be addressed within the structural context. We reasoned that a more detailed structure-guided engineering approach could reveal the precise structural elements of the protomer involved in the O-antigen chain length determination process. In view of the bell-shape architecture of the oligomers, it was also important to establish whether these regions would be found on the external surface of the oligomer or within the large, solvent-filled cavity. To this end, we engineered and tested over 90 different chimeric and mutant O-antigen chain length regulator proteins (Kalynych et al., 2011).

By examining the in vivo effects of the chimeric LPS O-antigen chain length regulators generated between two closely related WzzB of *S.typhimurium* and *S.flexneri*, we confirmed the importance of the C-terminal part of the molecule for the O-antigen chain length regulation. Within this large region, we discovered that a short stretch comprised of the amino acids 269–274 (inclusive) preceding the strand β4 could greatly alter the specification of the length of the growing polysaccharide chain. This region exhibits a certain degree of flexibility as it could not be modeled accurately in the structure of WzzB *S.flexneri* (Kalynych et al., 2012). It is situated at about ~25–30 Å away from the inner membrane where the O-antigen polymerization takes place. Moreover, most of the Wzy polymerases examined to date have been proposed to contain a large periplasm-exposed region (Kalynych et al., 2011). Thus, the significance of the bottom coil for the chain length determination can be rationalized by it serving as a possible interaction interface either with the Wzy polymerase or with the polysaccharide constituents of the growing O-antigen.

We also established that the regions making up the top of the molecules found at about ~100 Å away from the membrane are essential for the proper O-antigen maturation (Kalynych et al., 2011). Interestingly, modification of this region such as a swap between WzzB and FepE or a small deletion was sufficient to compromise the function of either chain length regulator. As is the case with 269:274 region, this segment of the protein was found to be disordered to different extents in the crystal structures of FepE and WzzB from *S.typhimurium* (Tocilj et al., 2008) underscoring its tendency to adopt multiple conformations.

Further structural investigation of the chimeric proteins revealed the architecture of the top part of the molecule (Kalynych et al., 2012). It was found to contain the top of the central α-helix as well as the long loops, which cap the oligomer. It is located some 100 Å away from the inner membrane and this distance is deemed too large to influence the polymerization process via direct interaction with the Wzy polymerase.

The possibility that the top of the oligomer interacts with an outer membrane component was quite intriguing. However, earlier studies showed that complete O-antigen assembly can take place in *S.typhimurium* spheroplasts, which are completely devoid of the outer membranes (Mulford and Osborn, 1983), suggests that such an interaction would not contribute to length selection but could play a role in transport to the outer membrane. Another possibility is the involvement of this region in binding of the O-antigen polysaccharide. The reasons for the importance of this part

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**Fig. 4.** The proposed model of O-antigen chain length regulation by the PCP-1 proteins. (A) Proposed mechanism of Wzy-catalyzed O-antigen polymerization. The polymerase is proposed to accommodate the incoming O-units and the polymerized O-antigen intermediate within two distinct sites (sites A and B, respectively). Over the course of the polymerization reaction the growing polysaccharide chain must be transferred to the newly accommodated undecaprenyl-pyrophosphate-linked O-unit (Und-PP O-unit). In the absence of the chain length regulator protein, the increase in length of the growing O-antigen favors its release before it can be transferred to the newly bound O-unit. (B) Chain length regulator proteins bind and stabilize the growing O-antigen intermediates in the conformations conducive to the continuous polymerization and thereby prevent the premature release of the growing polysaccharide. Once the O-antigen contains the number of the repeat units that can no longer be bound by the WzzB (or FepE) proteins, it is released.
of the molecule are not yet clear and require further investigation.

We were somewhat surprised to discover that we could not produce a functional protein by the crossover between the WzzB and FepE O-antigen chain length regulators imposing long and very-long O-antigen modal distribution, respectively. We found that even swapping a 19-amino acid glycine-and proline-rich region, which is proximal to the C-terminal transmembrane helix (TM), affected function. We concluded that such large-scale modification negatively influenced either the folding of the protein or the proper oligomer assembly. It was also somewhat surprising to discover that while the FepE periplasmic domain is fully functional when placed on the membrane and cytosolic portion of WzzB, the graft of the periplasmic portion of WzzB onto FepE is non-functional. The crystal structure of the full-length protein with the underlying architecture of the transmembrane regions and the cytosolic portions of the molecule are required to better understand these results.

Oligomeric structure of chimeric PCPs

We speculated that perhaps the observed differences in the O-antigen length between such closely related PCPs could be explained and correlated with the local conformational changes in selected regions. To further explore this possibility, we solved the crystal structures of several periplasmic domains of the hybrid WzzB proteins and that of the wild-type S. flexneri (Kalynych et al., 2012). Structural comparisons have shown that the protomers of all three molecules have nearly identical main chain confirmations. Unexpectedly, the chimeric chain length regulators were found to adopt the octamer arrangement. This oligomeric state was very different from the previously seen pentamers of the WzzB from S. typhimurium and from the open trimers of WzzB from S. flexneri. These results also differ from the work carried out on full-length proteins by Larue et al. (2009), who reported the hexameric organization for all LPS O-antigen chain length regulators examined. Maintaining the proper oligomeric state was shown to be important for establishing both long and very long O-antigen modal lengths (Papadopoulos and Morona, 2010; Kintz and Goldberg, 2011). Therefore, the ambiguity surrounding the real oligomerization state will have to be resolved, likely through the determination of crystal structures of the full-length proteins that will underscore the effect of TM helices on the oligomer size.

Are there physical interactions between the O-antigen biosynthesis proteins?

Various attempts have been undertaken to discover the protein–protein interaction network formed by the PCP proteins involved in the specification of the O-antigen length. While it was proposed that these proteins assemble into a multiprotein complex containing the flippase Wzx, the polymerase Wzy and the ligase, the experimental data to support this hypothesis are not clear. Carter et al. (2009) utilized immunoprecipitation of a chromosomally epitope-tagged Wzy and Wzz but failed to detect the presence of the protein–protein complex using this approach. Chemical cross-linkers were also used without much success to probe the presence of such a complex (M. Valvano, unpublished data). Recently, we completed yet another proteomic approach relying on the incorporation of the unnatural amino acids at specific positions throughout the WzzB protomer. By examining the cross-linking pattern of ~20 mutants and subjecting the higher molecular weight cross-linked products to the identification by mass spectrometry, we also failed to detect the presence of any of the aforementioned putative complex components (S. Kalynych and M. Cygler, unpublished data).

Recently, Woodward et al. (2010) reconstituted the pathway of O-antigen biosynthesis in vitro using the purified proteins. This study demonstrated that the length of the mature O-antigen depends solely on the presence of the Wzy polymerase and Wzz chain length regulator. Yet the interaction between the two proteins was not explicitly shown to occur over the course of the in vitro reaction. Previously, it was suggested by Guo and coworkers that Wzz could bind the polysaccharide based on the changes observed by the small angle scattering curves of the detergent-solubilized WzzB as well as by the circular dichroism spectroscopy (Guo et al., 2006; Tang et al., 2007). Structural characterization of the periplasmic domains of the chimeric chain length regulators also demonstrated that the regions influencing the specificity toward a given modal length distribution are rich in solvent exposed amino acid residues found in the regions where they could form the interactions with the growing chain (Kalynych et al., 2012). In addition, Daniels reported the co-immunoprecipitation of the O-antigen of Pseudomonas aeruginosa with the corresponding chain length regulators, strongly suggesting the existence of a polysaccharide co-polymerase:O-antigen interaction in vivo (Daniels et al., 2002).

These observations lead us to consider another model of the length control. Wzy polymerase must transfer the growing oligosaccharide to the non-reducing end of the single O-unit. As the O-antigens are getting increasingly longer, they probably tend to adopt higher order structures, which may interfere with the precise positioning of the sugar polymer within Wzy-binding site necessary for a glycosidic bond formation. We speculate that by binding the O-antigens, the chain length regulators maintain them in the confirmation permissive to further polymerization. Thus, while the growing O-antigen is interacting with the corresponding co-polymerase, the polymerization is continuing. As the O-antigens are achieving certain length they can no longer be bound by a given chain length regulator and simply dissociate. This change in polysaccharide confirmation is translated to the bottom of the Wzy-O-antigen complex and triggers their release from the Wzy polymerase making them available for a ligation reaction. The different modal lengths imposed by different O-antigen chain length regulators may stem from the subtle differences in interaction networks formed between the co-polymerase and the O-units comprising the polysaccharides. The reasons why the O-antigen polymers do not contain the precise number of the repeat units can be explained by the fact that even when bound by Wzz perhaps the carbohydrate polymers adopt various non-uniform higher order flexible structures which makes the polysaccharide–protein binding neither very strong nor highly specific, leading to a stochastic termination of their dissociation around certain oligosaccharide length and hence termination of elongation (polymerization). This model would also explain why the physical interaction between Wzy polymerase and the co-polymerases...
has not been observed in spite of numerous attempts (Carter et al., 2009 and M.A. Valvano personal communication) as it does not assume a physical interaction but rather their close proximity. This speculative model is further outlined in Fig. 4.

Based on the available crystal structures of the synthetic repeat units from S.flexneri 2a, it has been suggested that the mature O-antigen polymer would form a right-handed helix with a pitch of 23 Å containing nearly three repeat units per turn and a diameter of 15 Å (Vulliez-Le Normand et al., 2008). Extrapolating these dimensions on the polysaccharide containing 12 and 20 repeat units would yield 92 and 153 Å long polysaccharides, respectively. It is currently difficult to envision how the macromolecules of these dimensions are being handled in the periplasmic space of about 200 Å in width (Matias et al., 2003) containing a 60 Å-thick layer of the peptidoglycan cell wall (Yao et al., 1999). Further research efforts employing various multidisciplinary approaches will be necessary to uncover the missing pieces of the longstanding puzzle of precisely how the PCP family members regulate O-antigen maturation.

Funding
This work was supported by the Canadian Institutes of Health Research (CIHR) grant MOP-89787 to M.C. and M.A.V.

References