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Structure and Function of Lipid A Modifying Enzymes

By

Anandhi Anandan and Alice Vrielink

School of Molecular Sciences, University of Western Australia, 35 Stirling Highway, Perth,
WA, 6009, Australia.

Corresponding author: Alice Vrielink, School of Molecular Sciences, University of Western
Australia, 35 Stirling Highway, Perth, 6009, Australia.

Phone: +61 8 6488 3162,

Email: alice.vrielink@uwa.edu.au

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Abstract

Lipopolysaccharides are complex molecules found in the cell envelope of many Gram-negative bacteria. The toxic activity of these molecules has led to the terminology of endotoxins. They provide the microorganism with structural integrity and protection from external environmental conditions. Additionally, they interact with host signalling receptors inducing immune responses. Bacteria have evolved enzymes that act to modify lipopolysaccharides, particularly on the Lipid A region of the molecule, enabling the microorganism to circumvent host immune system responses. These modifications include changes to the endotoxin's charge by the addition of positively charged sugars, such as N-Ara4N, and the addition of phosphoethanolamine (pEtN). Other modifications include hydroxylation, acylation and deacylation of fatty acyl chains. In this review we give an overview the two-component regulation mechanisms for enzymes that carry out these modifications and provide details of the structures of 4 enzymes that act by modifying the Lipid A portion of lipopolysaccharides (PagP, PagL, pEtN transferases and ArnT). We focus largely on the three-dimensional structures of these enzymes which provide an understanding of how their substrate binding and catalytic activities are mediated. A structure-function based understanding of these enzymes provides a platform for the development of novel therapeutics to treat antibiotic resistance.

Introduction

The structure and tertiary fold of an enzyme dictates how it binds to its substrate and performs its catalytic reaction. Structure determination of enzymes provides a detailed understanding of the positions of amino acid residues, particularly around the active site and

enables hypotheses to be made concerning the catalytic mechanism. Understanding the structure and catalytic mechanism of enzymes that modify the structure of lipid A would provide valuable insights into deciphering new ways to combat or reverse highly virulent Gram-negative pathogens. They could form a basis for structure-based drug design which has become a powerful tool in the pharmaceutical industry. Structure-based drug design is an iterative process which uses the knowledge of the three-dimensional structure of a potential target such as an enzyme, to identify and optimize the interaction of a small molecule inhibitor to the target such that it will achieve at least micromolar binding affinity and specificity¹. The optimised lead compounds are taken to clinical trials and the successful compounds are then marketed as drugs for clinical use². Numerous drugs identified by structure-based drugs design technique, such as human immunodeficiency virus (HIV)-1 protease inhibitors,³ and thymidylate synthase inhibitors⁴ are successfully marketed for clinical use. The current review explores the three-dimensional structure and catalytic mechanism of four enzymes namely lipid A palmitoyltransferase (PagP), lipid A 3-O-deacylase (PagL), phosphoethanolamine (pEtN) transferase and aminoarabinose transferase (ArnT) that alters the structure of lipid A resulting in modified interaction with host recognition system and cationic antimicrobial peptide (CAMP) resistance in Gram-negative bacteria.

Lipopolysaccharides and Lipooligosaccharides

Gram-negative bacteria are characterised by the presence of a cell envelope comprised of an inner membrane, a peptidoglycan layer and an outer membrane (Figure 1 A). Lipopolysaccharides (LPS), also called endotoxins,⁵ make up the major component of the outer leaflet of the outer membrane and provide a barrier to the bacteria from the external

environmental conditions. These complex molecules, with their well conserved structure, also act as antigens to the host organism. In the event of an infection they are recognised as foreign molecules by the host immune system^{6, 7} generating a cascade of immune responses aimed at clearing the infection.

Differences exist between lipooligosaccharides (LOS) and LPS (Figure 1B). LPS is comprised of an inner hydrophobic lipid A moiety and an outer hydrophilic polysaccharide unit, also called the O-antigen, linked through a core oligosaccharide namely 3-deoxy-D-manno-octulosonic acid (keto-deoxyoctulosonate (KDO))⁸. Enteric bacteria characteristically present LPS; where the outer hydrophilic polysaccharide unit is long, complex and neutrally charged. On the other hand mucosal pathogens present LOS, characterised by shorter polysaccharide units⁹. The outer polysaccharide comprises a variety of monosaccharides residues (branched sugars, deoxy sugars, acid or amide forms of sugars, sugar phosphates and sugars substituted with amino acids, acylated or methylated at various positions) providing broad structural diversity of O-antigens in Gram-negative bacteria¹⁰. On the other hand the core oligosaccharides and the lipid A region of this complex molecule is relatively well conserved among all Gram-negative bacteria⁷ and it is this region that is responsible for the cytotoxic properties of the molecule⁵. The lipid A structure contains a 1,4'-bisphosphorylated- β -D-glucosaminyl-(1 $\rightarrow$ 6)-D-glucosamine disaccharide backbone with 4 to 7 fatty acid chains attached to the N-acetylglucosamine sugars. Different strains of Gram-negative bacteria exhibit fine variations in the lipid A structure which arises mainly from the nature, chain length, number, location of acyl groups, types of hexosamines present, the degree of phosphorylation and the presence of substituents such as pEtN and 4-amino-4-deoxy-L-arabinose (L-Ara4N)¹¹.

Generally, LPS/LOS are negatively charged due to the presence of phosphate moieties and acidic sugars. This negative charge can lead to electrostatic repulsion between the LPS/LOS

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molecules assembled in the outer membrane of Gram-negative bacteria and weaken the outer membrane. In order to maintain membrane integrity, bacteria sequester divalent cations, mainly Mg^{2+} , to neutralise the negative charge and reduce this repulsion¹². Furthermore, stability of the outer membrane is maintained by lateral interaction arising from hydrogen-bond donors and acceptors from the lipid A moiety. Along with this, the high number of acyl chains per molecule of lipid A increases the hydrophobic space and reduces fluidity of the outer membrane¹³. The tight lateral interactions between LPS/LOS coupled with the low membrane fluidity enables the bacterial membrane to provide a permeability barrier to lipophilic solutes and detergents⁵.

Bacterial infection and the host immune system

Effective infection depends on a dynamic interaction between the microbial pathogen and the host. Factors such as variation in the bacterial virulence factor profile, their metabolism, and host innate immune defences play a major role in bacteria-host interaction and disease progression¹⁴. Bacteria are capable of altering the expression of their virulence genes depending on the environment towards infecting a host.

The overall structure of lipid A is conserved in all Gram-negative bacteria, providing a molecular pattern for the host cell receptors to elicit an immune response. LPS/LOS released from the bacterial surface during an infection activate the host innate immune system by binding to LPS-binding proteins to initiate a signalling event^{15, 16}. The LPS/LOS bound LPS-binding protein interacts with CD14 proteins on the surface of myeloid cells resulting in the transfer of LPS/LOS to CD14¹⁷. The LPS/LOS is then transferred to the signalling receptor complex: myeloid differentiation factor 2 (MD2)/toll-like receptor 4 (TLR4), which results in a

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3 cascade of signalling pathways (Figure 3B)^{17, 18}. The number and length of acyl chains impacts
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5 the hydrophobic interaction of LPS with MD2 and induces homo-dimerization of the
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7 TLR4/MD2 complex influencing the intracellular response level¹⁸. A hexa-acylated lipid A with
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9 side chains of 12 to 14 carbons in length has been shown to provide the optimal level of
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11 inflammatory response¹⁸⁻²⁰. Furthermore, the presence of phosphate groups at the 1 and 4'
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13 positions of lipid A enhance interactions with positive amino acid residues in TLR4/MD2 aiding
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15 in homo-dimerization^{18, 21}.
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19 This formation of the homodimeric TLR4/MD2 complex, and the initiation of the signalling
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21 cascade, leads to the production of CAMPs²²⁻²⁴, a variety of cytokine and chemokine
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23 molecules (Figure 3B)²⁵, and signals the presence of an infection to cells of the host's adaptive
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25 immune system²⁶. These responses provide antibacterial defences, essential for controlling
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27 and clearing the infection. CAMPs form the first-line of defence against invading microbes²⁴.
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29 They generally consist of short amphipathic molecules with a net positive charge²⁷ and exhibit
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31 broad-spectrum antimicrobial activity with bacteriostatic, bactericidal and cytolytic
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33 properties²⁸.
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37 The cationic nature of CAMPs enable them to competitively displace the LPS/LOS-associated
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39 divalent cations and interact with the anionic head group of lipid A located in the bacterial
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41 outer membranes^{27, 29, 30}. This leads to a significant conformational change in the CAMP^{30, 31}
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43 and reveals the amphipathic nature of the peptide³². The amphipathic structure facilitates the
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45 insertion of CAMPs into the membrane and the polar face of the peptide interacts with the
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47 phosphate groups in the lipid bilayer forming an initial strong electrostatic interaction³³. This
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49 interaction destabilises the membrane allowing the lipophilic face of the CAMP to insert into
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51 the hydrophobic fatty acyl membrane layer^{13, 34, 35} before uptake into the periplasmic space³⁶.
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Ultimately CAMPs target the inner membrane, where they kill bacterial cells primarily by disrupting the integrity of the membrane³⁷ and the membrane potential^{36, 38}.

Hence the ability of the host to sense the presence of an infection, and the interaction of CAMPs with the bacterial membrane aimed at perturbing the membrane structure, are important requirements for bactericidal activity. The conserved structure of lipid A along with both the hydrophobic and charged nature of the LPS/LOS facilitates recognition of an infection and strong binding of CAMPs to the bacterial membrane³⁷ respectively. However, Gram-negative bacteria have evolved mechanisms to modify the structure of LPS/LOS in different environments^{13, 29, 30}, which alter recognition by the TLR4/MD2 receptor complex and promote resistance to host CAMPs.

PhoPQ, a two-component regulatory system in Gram-negative bacteria (Figure 2), allows them to sense changes in the environment and respond to these changes by regulating the expression of countermeasures needed for survival of the pathogen³⁹. The presence of CAMPs at the bacterial membrane surface alters the Mg^{2+} ion concentration. Reduced Mg^{2+} ion concentration at the membrane surface is sensed by the PhoQ protein of the PhoPQ regulatory system. PhoQ is a histidine kinase found in the cytoplasmic membrane with an external loop on the periplasmic side and a conserved histidine residue on the cytoplasmic side⁴⁰. The external periplasmic loop senses changes in the Mg^{2+} ion concentration through a change in the protein conformation followed by auto phosphorylation of the conserved histidine residue on the cytoplasmic facing surface of the protein³⁹. The phosphorylated PhoQ then transfers the phosphate to the PhoP protein which in turn activates the transcription of the PhoP activated gene (*pag*) and represses the PhoP repressed gene (*prg*)⁴¹. Expression of *pag* can further activate another two-component system, specifically PmrAB, leading to expression of PmrA dependent genes^{39, 42-44}. Again PmrB is a kinase sensor while PmrA is a

response regulator; this system either gets activated by the PhoPQ system or independently through responses to changes in the bacterial growth environmental such as a decrease in pH⁴⁵ or increased Fe³⁺ ion concentration⁴⁶. Both the PhoPQ^{47, 48} and PmrAB^{42, 43, 49} two-component signal transduction pathways regulate the expression of a number of enzymes that modify the structure of lipid A. Lipid A modifications contribute to variations to the LPS/LOS structure and a number of the enzymes responsible were first characterised in *S. typhimurium* and *E. coli*⁵⁰. Subsequently these enzymes have also been identified in other human pathogens as well as commensal organisms.

Lipid A diversity and implications

Structural changes in lipid A include the addition of palmitate, L-Ara4N and pEtN as well as hydroxylation and deacylation of fatty acyl chains resulting in an outer membrane composed of a mosaic of more than 20 individual lipid A structures depending on the species of Gram-negative bacteria (Figure 3). Neutralisation of negative charges in lipid A by modifying the phosphates with positively charged L-Ara4N and/or pEtN substituents provides resistance to CAMPs by blocking the initial electrostatic interactions⁵¹. ArnT and pEtN transferase are two enzymes expressed under the control of the PmrA/PmrB two component regulatory system (Figure 2). These enzymes modify the 1 and 4' positions of lipid A with L-Ara4N and pEtN respectively⁴⁹ (Figure 3). The addition of pEtN decreases the net charge of the bacterial surface from -1.5 at neutral pH to -1 and increases the lateral interaction of LPS/LOS in a more specific manner¹³ leading to decreased affinity to CAMPs binding and increased resistance. Depending on the bacterial species, the modification of lipid A with pEtN can result in the replacement of a phosphate group at the C-1 position, as reported in *H. pylori*⁵², or the

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addition of pEtN to the phosphate groups at the C-1 and C-4' positions or both as reported in pathogenic *Neisseria spp.*⁵³. Furthermore, removal of the phosphate group or alteration of the negative charge, through chemical modification of the phosphate, also affects the interaction between the TLR4/MD2 receptor and lipid A¹⁸ resulting in a reduced immune response.

In addition to neutralizing the charge of the phosphate groups, the greatest modification of lipid A is through acylation. The outer membrane of Gram-negative bacteria provides a significant barrier to CAMP uptake and changes in the number or length of acyl chains in lipid A alters the hydrophobic and van der Waals interactions affecting the fluidity of the outer membrane. This in turn interferes with CAMP translocation across the bilayer⁵⁴. Alterations in acylation are brought about by three enzymes which are regulated by the PhoPQ two-component regulatory system. The reactions catalysed by enzyme lipid A palmitoyl transferase (PagP), lipid A 3-O-deacylase (PagL) and dioxygenase (LpxO) lead to the acylation, deacylation and hydroxylation of Lipid A molecule respectively (Figure 3). A list of gene expressing enzymes modifying lipid A that are conserved in various Gram-negative pathogens are presented in Table 1.

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Structure of PagP

PagP is an integral outer membrane acyltransferase that catalyses the transfer of a palmitoyl group from the *sn*-1 position of a glycerophospholipid to the N-linked R-3-hydroxy myristate chain on the proximal glucosamine unit of lipid A resulting in palmitoylated lipid A and lysophospholipid (Figure 3)⁵⁵. The *pagP* gene responsible for lipid A palmitoylation was first identified in a *Salmonella* mutant that was found to be sensitive to CAMPs⁵⁴. The structure of PagP from *E. coli* has been elucidated by both Nuclear Magnetic Resonance (NMR)

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spectroscopy⁵⁶ and X-ray crystallography⁵⁷. PagP has an N-terminal α -helix followed by an 8 stranded antiparallel β -sheet (strand A to H (Figure 4A)) forming a barrel in the outer bacterial membrane^{56, 57}. The strands are connected by short loops at the periplasmic end and longer, more flexible loops on the extracellular surface of the protein^{56, 57}. PagP spans across the outer membrane with an approximate 25° axis tilt relative to the membrane surface (Figure 4A) aligning the residues that comprise the aromatic belt at the membrane interface⁵⁸. The N terminal α -helix is amphipathic lying at the membrane interface on the periplasmic side and makes contacts with the end of strands B and C. This contact fills the space caused by the tilt of the main barrel on the periplasmic side while a β -bulge at Pro28 near the end of strand A occupies the space on the extracellular side⁵⁷.

PagP selectively transfers a single 16-carbon acyl-chain from a phospholipid donor to lipid A. The crystal structure reveals a single lauroyl-dimethylamine-N-oxide (LDAO) molecule bound to the PagP β -barrel interior, with its polar head group facing the extracellular region and acyl chain extending into the core of the β -barrel (Figure 4A). The bound LDAO molecule suggests that the substrate binding pocket of PagP is situated within the barrel, projecting towards outer half of the barrel structure. The extracellular facing side of the barrel is lined with hydrophobic residues while the intracellular facing side has hydrophilic residues⁵⁷. The side chains of the aromatic residues in the substrate binding pocket along with Gly88, positioned at the floor of the hydrophobic region, act as a hydrocarbon ruler, defining specificity for a sixteen-carbon palmitate substrate⁵⁹ (Figure 4B). In contrast, PagP lacks acceptor substrate specificity and can transfer palmitate to various fatty alcohols as demonstrated by an *in vitro* activity assay⁵⁵.

Three conserved residues: His33, Asp76 and Ser77, located in the extracellular loop above the hydrocarbon ruler, with their side chains oriented towards the axis of the β -barrel are thought

to be the active site residues forming a catalytic triad and mutational studies of these residues proved that they were essential for the enzyme activity⁵⁶. However, the crystal and NMR structures show that His33 on loop1 is well separated from Asp76 and Ser77 on loop2⁵⁶. Alternatively, it is thought that PagP catalysis could occur through formation of a ternary complex with both the substrates accessing the interior of the β -barrel. This would enable a direct transfer of the palmitoyl group from the phospholipid donor to the lipid A acceptor without the formation of an acyl-enzyme intermediate⁶⁰.

NMR studies of PagP reports that the enzyme is dynamic and alternates between a tensed state and relaxed state⁶¹. Loop 1 and the adjacent region in PagP adopts a relaxed form facilitating substrate entry laterally from the membrane phase while the tensed form facilitates catalysis. Interestingly, a number of Proline residues found in PagP disrupt the hydrogen bonds within the β -barrel structure. The presence of Pro28 in strand A and Pro50 in strand B beneath loop 1 weaken the hydrogen bonds between these two strands and are termed as a molecular embrasure. Pro127 in strand F and Pro144 in strand G weaken the hydrogen bond between these strands causing an indentation in the β -barrel and are termed as a molecular crenel⁶². These fissures in the barrel may allow both the donor substrate and the acceptor substrate to laterally diffuse into the barrel through the crenel and the embrasure respectively⁶² (Figure 4C). Furthermore, the presence of Lys42 on the loop 1 surface has been reported to play a role in Lipid A recognition⁶².

Structure of PagL

PagL, an enzyme expressed from the PhoPQ activated gene, *pagL*, hydrolyses an ester linkage at the 3 position of lipid A and removes an acyl chain (Figure 3)⁴⁸ conferring CAMP resistance⁶³. PagL activity occurs only when lipid A lacks L-Ara4N substitution^{64, 65} even

though the expression of both PagL and ArnT genes are controlled by the PhoPQ two-component system. Since deacylation of lipid A increases the fluidity of the membrane, leading to increased permeability, PagL latency provides the bacterium with a robust membrane while growing in PhoPQ activating conditions⁶⁶. Furthermore, 3-O-deacylated lipid A has been shown to have lower affinity to the TLR4/MD2 receptor resulting in a reduced immune response in comparison to hexa acylated lipid A^{67, 68}. A PagL homologue is found in a number of Gram-negative bacteria but the sequence identity between them is low⁶⁹.

PagL is an outer membrane protein and was first discovered in *S. typhimurium* but is absent in *E. coli*⁴⁸. The crystal structure of PagL from *Pseudomonas aeruginosa* was solved to 2.0 Å resolution revealing substrate binding features of the enzyme and providing an understanding of the catalytic mechanism⁷⁰. The overall structure of PagL consists of an eight-stranded antiparallel β-barrel with both the amino and carboxy termini facing the periplasm. PagL has a tilted orientation in the membrane stabilised by two belts of aromatic residues aligned at the membrane interfaces (Figure 5). The β-strands of PagL are linked by long extracellular facing loops (L) and short periplasmic facing turns (T)⁷⁰. Although sequence identity of homologues from various Gram-negative bacteria is low, four highly conserved amino acid residues are evident: Phe104, His126, Ser128 and Asn136⁶⁹. In *P. aeruginosa* His126 and Ser128 along with Glu140 (despite its lack of conservation in other homologues) form the catalytic triad and these residues are located near to L4 on the extracellular facing side of the β-barrel⁷⁰. Asn129 has been shown to play an important role in stabilizing the conformation of L4 and the catalytic triad⁷⁰. The catalytic mechanism of PagL is similar to that of serine hydrolase⁶⁹. Glu140 acts as a base to deprotonate His126, which in turn abstracts a proton from Ser128 enabling a nucleophilic attack on the carbonyl carbon of the scissile bond.

Ala130, Gly131 and Asn136 form the oxyanion hole to stabilise the transient negative charge on the carbonyl oxygen of the substrate during the reaction⁷⁰.

Modelling and energy minimization of PagL with the lipid X substrate revealed a conserved residue, Phe104, found in the hydrophobic groove, positions the acyl chain to be cleaved closer to the catalytic Ser128 for nucleophilic attack. The side chain of Asp106 situated on the opposite side of His126 points towards the hydrophobic region of the membrane and forms a hydrogen bond with the hydroxy group of this acyl chain enabling substrate selectivity⁷⁰. As mentioned earlier, PagL removes the acyl chain from the third position on lipid A but lacks specificity in terms of the length of the acyl chain⁷¹.

PagL from *P. aeruginosa* and PagP from *E. coli* are both outer membrane proteins with eight-stranded antiparallel β -barrel but their substrate binding sites and their ability to distinguish acyl chains based on chain length are quite different. Unlike PagP the substrate binding site of PagL is found on the outer surface of the β -barrel⁷¹.

pEtN transferase

pEtN transferase transfers pEtN from the donor substrate, phosphatidylethanolamine, to the acceptor substrate, LPS/LOS, at various locations depending on the specific enzyme. A phylogenetic analysis of predicted pEtN transferases showed six main families of the enzyme depending on the location of pEtN substitution on LPS/LOS⁷². There are pEtN transferases specific to lipid A⁵³, the 3 and 6 positions of the second heptose in the inner core region of LPS/LOS^{73, 74}, the galactose residue in LPS, the KDO, as well as proteins on bacterial surface such as the flagella and pilin structures⁷⁵. A number of pEtN transferases have been identified in Gram-negative bacteria (*E. coli*⁷⁶, *S. enterica*⁴⁹, *N. meningitidis* and *N. gonorrhoea*^{53, 74, 77}).

Some Gram-negative bacteria possess multiple genes to express different pEtN transferases specific for the addition of pEtN to different locations on LPS/LOS while others express a single pEtN transferase that adds pEtN to different locations on LPS/LOS. *Haemophilus ducreyi* has three genes *lptA*, *ptdA* and *ptdB* and catalyses pEtN modifications at two different sites on the acceptor LOS molecule: a pEtN transfer onto lipid A and a pEtN transfer onto the KDO moiety of its core oligosaccharide⁷⁸. pEtN transferases, CptA and EptB modify the heptose II or KDO core sugars of the LPS in *S. enterica* and *E. coli* respectively^{49, 76}. Pathogenic *Neisseria spp.* have three pEtN transferases that add pEtN to LOS. NmEptA modifies the lipid A while Lpt-3 and Lpt-6 add pEtN to the heptose II core sugar at the third or sixth position respectively^{53, 79, 80}. In contrast, a single pEtN transferase, from *Camphylobacter jejuni* (CjEptC) is responsible for modification of lipid A, the first heptose residue of the inner-core oligosaccharide of LOS and Thr75 of the flagellar rod protein, FlgG, with pEtN^{75, 81}. More recently a plasmid mediated mobile colistin resistance gene (*mcr-1*) has been discovered in *E. coli* initially infecting pigs and poultry and now also identified in *E. coli* which infect humans. Colistin is a cationic polypeptide antibiotic that is used as a last-resort to treat infections caused by multidrug-resistant Gram-negative pathogens⁸² while Mcr-1 is a pEtN transferase that adds pEtN to 1 and 4' glucosamine moiety of lipid A conferring colistin resistance to these organisms⁸³. Unlike the other pEtN transferase genes that are chromosomal, the gene coding for MCR-1 is located on a plasmid and can be transmitted from one species to another⁸⁴ rapidly spreading colistin resistance. More recently, multiple variants of *mcr-1* genes namely *mcr-2*⁸⁵, *mcr-3*⁸⁶ and *mcr-4*⁸⁷ have been identified in various strains of Gram-negative bacteria.

pEtN transferases are metalloenzymes belonging to the alkaline phosphatase superfamily and contains a hydrophobic transmembrane domain embedded in the cytoplasmic

membrane and a globular C-terminal domain with a hydrolase fold⁵³ presented on the periplasmic side of the bacterial membrane. They all have conserved metal-binding residues as well as a conserved catalytic threonine or serine⁸⁸.

The atomic structure of the soluble C-terminal domain of pEtN transferase from *N. meningitidis* (NmEptA) was solved to 1.43 Å resolution⁸⁹ initially followed by the structure of full length NmEptA⁹⁰. Subsequently, the structures of the soluble domain of pEtN transferase from *C. jejuni* (CjEptC)⁹¹, *E. coli* (MCR-1 and EcEptC)^{92, 93} and *Moraxella catarrhalis*⁹⁴ have also been resolved. CjEptC, EcEptC and MCR-1 are all homologues to NmEptA and exhibit a high degree of structural similarity. Here we focus on the structure of NmEptA while highlighting the differences in the other pEtN transferase structures as required.

The structure of NmEptA is composed of two discretely folded domains: a C-terminal soluble domain and an N-terminal transmembrane (TM) domain connected by a bridging helix and an extended loop (Figure 6A). The periplasmic domain of pEtN transferase adopts a $\alpha/\beta/\alpha$ hydrolase fold. NmEptA, EcEptC and MCR-1 have seven β strands sandwiched by eleven α helices^{89, 93, 95, 96} while CjEptC has six β strands surrounded by eleven α helices⁹¹. A Zn^{2+} ion is tetrahedrally coordinated to the side chains of conserved residues: His453, Asp452, Glu240 and Thr280^{89, 90} in NmEptA. A phosphate group is covalently linked to the conserved Thr280 in the soluble domain structure. Both the phosphorylation and superimposition of other nearest structural homologues suggest that this threonine side chain is the catalytic nucleophile required for the transfer of pEtN^{89, 91, 92}. Mutational and mass spectrometry studies by Fang *et al* have shown that replacing the conserved threonine with serine resulted in the absence of a covalently linked phosphate⁹¹. The crystal structures of the soluble domains of CjEptC and MCR-1 have captured the phosphorylated threonine while this is not the case in the EcEptC structure⁹³. The phosphorylated Thr280, along with the tetrahedrally

coordinated Zn^{2+} ion, was identified as the active site region of the enzyme. Structural homology studies of NmEptA suggests that Zn^{2+} ion is important for catalysis and is likely to stabilise the nucleophilic alkoxide on Thr280⁸⁹. A number of disulphide bonds are found, stabilising flexible loops in the structure. NmEptA has 10 cysteines in the soluble domain forming 5 disulphide bonds (Figure 6B) while CjEptC and MCR-1 have 3 disulphide bonds. Intriguingly, EcEptC does not have any cysteine residues in the soluble domain to form disulphide bonds⁹³. Moreover, while both are found in *E. coli*, EcEptC is inactive *in vivo* while MCR-1 is active. Zhao *et al* have also shown that the crystal structure of the soluble domain of EcEptC lacks the tetrahedrally coordinated Zn^{2+} ion irrespective of presence of the conserved Zn^{2+} binding residues. Together they propose that the disulphide bonds could play an important structural role in restraining the flexibility of the loops and assisting in holding the active site residues in position for binding of the Zn^{2+} ion⁹³. A structure-based sequence alignment⁹⁷ of NmEptA, CjEptC, MCR-1 and EcEptC showing the positions of the disulphide bonds is provided in Supplementary Figure 1.

As mentioned earlier, NmEptA have two extra disulphide bonds in comparison to CjEptC. One of them is found in a region where the sequence differs significantly between NmEptA and CjEptC while the second one is found in the loop located near to the active site. This loop, which is shorter in CjEptC than in NmEptA, is thought to play a role in acceptor substrate specificity in NmEptA⁹¹. In contrast, MCR-1 also has only three disulphide bonds but one of them differs from the three disulphide bonds found in CjEptC. MCR-1 has a disulphide bond in the extended loop near the active site, similar to NmEptA and exhibits acceptor substrate specificity⁹⁶.

The structure of full length NmEptA reveals a membrane domain that contains five transmembrane helices (TMH1 to TMH5) spanning the cytoplasmic membrane (Figure 5A).

These five transmembrane helices are connected by loops some of which harbour smaller helices at the periplasmic side of the membrane bilayer. The membrane domain contains two tryptophan residues and a number of tyrosine and histidine residues forming an aromatic belt along the membrane surface allowing a prediction of the membrane spanning regions⁹⁸ of EptA. The five transmembrane helices are oriented approximately parallel to one another and only TMH5 fully spans the width of the bilayer with a length of 35 Å; the other four TM helices are shorter than the typical width of a membrane bilayer (30 Å). A patch of positively charged residues at the cytoplasmic end of TMH5 provides interactions with the phospholipid head groups at the inner membrane surface. Based on the positions of the aromatic belt residues and positively charged residues, TMH5 is said to have a tilt to compensate for the hydrophobic mismatch^{99, 100} between the TMH5 and the membrane.

The membrane domain is connected to the soluble domain by a bridging helix and an extended periplasmic loop. The bridging helix has a highly unusual orientation in the structure. It is oriented parallel to the membrane surface with hydrophobic residues at the N-terminal portion and charged residues at the C-terminal region. In the membrane domain, a pair of short periplasmic facing helices PH2 and PH2' are found in the loop that connects TMH3 and TMH4 (Figure 6C). These periplasmic helices are amphipathic with polar residues facing the periplasm and the nonpolar residue directed towards the membrane suggesting that these two helices could be partially embedded in the membrane⁹⁰.

The soluble domain from the structure of full length NmEptA has a fold very similar to the previously described structure with no significant differences except for the presence of a dodecyl-β-D-maltoside (DDM) detergent molecule at the active site⁹⁰. The first carbohydrate moiety of the DDM lies deep in the substrate pocket with a hydroxyl group in close proximity

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3 to the bound Zn^{2+} ion and to the active site nucleophile Thr280. The distal portion of the DDM
4 molecule extends between the PH2 and PH2' helices. Thus the structural arrangement of the
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6 molecule extends between the PH2 and PH2' helices. Thus the structural arrangement of the
7
8 two domains reveals a sequestered active site⁹⁰. The presence of the bound DDM molecule
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10 has been proposed to mimic the phosphatidylethanolamine bound state of NmEptA with the
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12 two domains making a closed structure. However, this conformation does not facilitate
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14 binding of a large lipid substrate such as LOS. As the enzyme is proposed to form a pEtN -
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16 enzyme intermediate with pEtN linked to Thr280, and undergo its kinetic chemistry through
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18 a ping pong mechanism, it may be that the molecule may undergo a conformational change
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20 to facilitate binding of the acceptor LOS substrate and transfer of pEtN from the enzyme
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22 intermediate. A molecular dynamic study supports this hypothesis showing conformational
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24 flexibility in terms of the relative orientation of the two domains⁹⁰.
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34 Structure of ArnT

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36 ArnT is a member of the GT-C glycosyltransferase family¹⁰¹ and attaches a cationic sugar, L-
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38 Ara4N, to the phosphate groups at the 1 and 4' positions of lipid A (Figure 3). UDP-glucuronic
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40 acid is converted to L-Ara4N in the cytoplasm by a set of enzymes, ArnA, ArnB, ArnC and ArnD,
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42 and is then linked to the lipid carrier, undecaprenyl phosphate (UndP)⁵⁰. UndP-linked L-Ara4N
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44 is flipped across the cytoplasmic membrane and the L-Ara4N moiety transferred to lipid A by
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46 ArnT¹⁰². The addition of L-Ara4N to the inner core sugar (D-glycero-D-talo-octo-2-ulosonic
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48 acid) is also observed in certain Gram-negative bacteria such as *Burkholderia cenocepacia*¹⁰³
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50 and *Serratia marcescens*¹⁰⁴. *Proteus* strains attach L-Ara4N to KDO¹⁰⁵ while *Ralstonia*
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52 *solanacearum* catalyse the addition to L-glycero-D-manno-heptose (Hep)¹⁰⁶ residues of the
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54 core oligosaccharide.
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The crystal structures of ArnT in the unbound and UndP-bound forms from *Cupriavidus metallidur* were solved to 2.8Å and 3.2Å respectively¹⁰⁷. ArnT has a transmembrane domain with 13 transmembrane helices and a periplasmic domain¹⁰⁸ with an $\alpha/\beta/\alpha$ fold. It has a six stranded β -sheet surrounded by α helices as shown in Figure 7A. An extended loop on the periplasmic side joins TMH1 and TMH2 and this loop harbours 2 periplasmic helices (PH1 and PH2) that lie perpendicular to each other. A third periplasmic helix (PH3) is found on the periplasmic loop (PL4) that connects TMH7 and TMH8 and has been shown to play an important role in substrate binding and/or transfer of L-Arn4N to lipid A¹⁰⁹. ArnT has 8 cysteine residues in their sequence but they are not positioned to enable disulphide bond formation, and are not required for structural stabilisation as observed in EptA¹⁰⁹. Three major cavities were observed in the structure of ArnT with the largest one, cavity 1, amphipathic in nature. This cavity has a hydrophobic region lying below the proposed membrane surface and a hydrophilic region above the membrane surface, capable of accommodating the acyl chains and glucosamine backbone respectively. However, passage of ligands into this cavity is obstructed by PL4 in the apo-ArnT structure. A Zn²⁺ ion coordinated by Glu84 and two histidine side chains, His265 and His267, located at the PH3 and PL4 regions of the structure, fix the loop confirmation¹⁰⁷. Cavity 2 is connected to cavity 1 through a narrow hydrophilic passage. This cavity has density to accommodate the phosphate head group and five prenyl groups of UndP in the ligand bound structure, identifying the location of the active site. The phosphate of UndP is coordinated by lysine and arginine residues in the narrow hydrophilic passage and the oxygen of the phosphodiester bond participates in hydrogen bonding with Tyr345 (Figure 7B). Cavity 3 on the other hand is hydrophobic and located near to the cytoplasmic side of the molecule¹⁰⁷. The last four prenyls of UndP extend from cavity 3 in the UndP bound structure suggesting that the lipid tail of

UndP must be binding to the hydrophobic residues in this cavity. A structural rearrangement of PL4 from a coil to a helix is observed in the UndP-bound structure and the transition not only leads to a loss of Zn^{2+} coordination but also displaces the PL4 loop away from the cavity 1 surface enabling lipid A access¹⁰⁷ (Figure 7C). Thus, the binding of substrates to ArnT may be sequential with UndP- α -L-Ara4N binding to the protein and stabilizing a conformation accessible to lipid A¹⁰⁷.

Although both ArnT and EptA are integral membrane proteins and modify the same site on lipid A, their structures and substrate binding mechanisms are distinctly different from one another. ArnT has two separate substrate cavities and the active site is situated in between them enabling the transfer of L-Ara4N directly from UndP- α -L-Ara4N to lipid A. In contrast, the structure of EptA is flexible suggesting a ping pong mechanism for transfer of pEtN to lipid A via an enzyme intermediate.

Conclusion

The structure and function of enzymes modifying the Lipid A structure have adapted to provide the invading pathogen mechanisms to escape the host innate immune system and establish an infection. By altering the charge or hydrophobicity of the outer membrane an altered binding affinity between the CAMP and the outer membrane can be established. The importance of lipid A modifying enzymes in bacterial pathogenesis suggests that knowledge of their structure-function relationships might lead to the development of novel inhibitors to these enzymes for treating bacterial infections.

Current public health is highly challenged due to the rapid development of multidrug resistant pathogens, resulting in an urgent need for the development of new strategies to combat the

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problem. One such strategy is to focus on novel therapeutic agents that inhibit bacterial virulence rather than bacterial viability¹¹⁰. Moreover, antimicrobial peptides are now receiving increasing attention for clinical antibacterial applications¹¹¹ especially against multidrug resistant bacteria. Inhibiting Lipid A modifying enzymes from Gram-negative bacteria will not only improve the efficacy of these antimicrobial peptides but also provide a range of pharmacological targets to generate new antimicrobials. Hence understanding the structure-function relationship of Lipid A modifying enzymes will shed light on developing inhibitors to these enzymes and strategies to meet the challenges of antibiotic resistance.

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Figure Legends

Figure 1

(A) Representation of the cell envelop of Gram-negative bacteria showing the inner membrane, the outer membrane and the periplasmic space containing the peptidylglycan layer. The constituent molecules found in the different structures of the cell envelop are shown. (B) Architecture of LPS and LOS in Gram-negative bacteria. The sugar molecules are represented by hexagons with GlcN: N-acetylglucosamine, KDO: 3-deoxy-D-manno-octulosonic acid (keto-deoxyoctulosonate), Hep: heptose, Gal: galactose and Glu: glucose. Phosphates are represented by a green circle labelled P.

Figure 2

Schematic representation of the activation and interaction of PhoPQ and PmrAB two component system leading to enhanced production lipid A modifying enzymes. P denotes phosphate.

Figure 3

(A) Chemical structure of Lipid A showing the enzymes that carry out modifications to the lipid substrate and the sites where modifications occur. (B) Schematic overview of innate immune signaling by LPS/LOS leading to production of CAMPs, cytokines and co-stimulatory molecules.

Figure 4

(A) Secondary structure representation for PagP (PDB: 1THQ) in the presence of lauroyl-dimethylamine-N-oxide (LDAO). The N terminus of the enzyme is coloured blue and the C terminus is coloured red. The strands making up the β -barrel are labelled as A to H. The bound LDAO molecule is shown as van der Waals spheres. Proposed active site residues (His33, Ser77 and Asp76) are shown in magenta stick representation. A loop between strands A and B is not evident in the structure. The membrane surface is depicted as a grey line and the residues comprising the aromatic belts at the membrane interfaces are shown in grey stick representation. (B) Secondary structure of PagP showing the amino acid side chains that contact the bound LDAO ligand and play a role as a hydrocarbon ruler to facilitate a sixteen-carbon acyl chain to bind inside the beta barrel structure. The LDAO molecule is shown in blue stick representation with a dot van der Waals surface and the amino acid side chains are shown in cyan coloured sticks. (C) Depiction of PagP showing the sites of substrate entry. Entry of phosphatidylethanolamine occurs through a molecular crenel in the structure and entry of LPS/LOS occurs through a molecular embrasure. Proline residues that affect the barrel structure at these two openings are indicated.

Figure 5

Secondary structure representation for PagL (PDB: 2ERV). The N terminus is coloured blue and the C terminus is coloured red. The strands making up the β -barrel are labelled as A to H. Extracellular loops are labelled as L and periplasmic facing turns are labelled as T. Proposed active site residues (Glu140, His 126 and Ser128) are shown in magenta stick representation.

The membrane surface is depicted as a grey line and the residues comprising the aromatic belts at the membrane interfaces are shown in grey stick representation.

Figure 6

(A) Secondary structure representation of EptA from *Neisseria meningitidis* (PDB: 5FGN). The N terminus is coloured blue and the C terminus is coloured red. The two domains are labelled as TM for transmembrane domain and SD for soluble domain. The bridging helix is labelled as BH and the transmembrane helices are labelled and numbered as TMH1 through TMH5. The membrane surface is depicted as a grey line and the residues comprising the aromatic belts at the membrane interfaces are shown in grey sticks. The bound DDM molecules is shown in ball and stick representation. Inset depicts a close up of the active site with the side chains included for residues that coordinate the Zn²⁺ atom (Glu240, Asp452 and His453) as well as the proposed nucleophile, Thr280 shown in white stick representation. (B) Close up of the soluble domain of EptA showing the positions of the disulphide bonds as yellow sticks. (C) Close up of the proposed substrate binding region of EptA. The periplasmic facing helices, PH2 and PH2', are colored in light blue. The bound DDM is shown in ball and stick representation. The Zn²⁺ ion is shown as a grey sphere.

Figure 7

(A) Secondary structure representation of ArnT from *Cupriavidus metallidur* (PDB:5EZM) in the apo form and (B) in the UndP bound form (PDB: 5F15). The N termini are coloured blue and the C termini coloured red. The two domains are labelled as TM for transmembrane domain and SD for soluble domain. The transmembrane helices are labelled and numbered

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3 as TMH1 through TMH12. The periplasmic helix, PH3, is indicated as is the periplasmic facing
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5 loop PL4. The membrane surface is depicted as a grey line and the residues comprising the
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7 aromatic belts at the membrane interfaces are shown in grey sticks. Inset of panel A shows a
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9 close up of the Zn^{2+} binding site with the metal shown as a grey sphere, and coordinating
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11 residues (Glu84, His265 and His267) represented as white sticks. Inset of panel B shows the
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13 close up of the UndP binding site with side chains of residues (Lys85, Arg278 and Tyr345)
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15 implicated in ligand binding shown in white sticks. (C) Superposition of the apo and UndP
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17 bound ArnT structures. The apo structure is coloured pink and the UndP-bound structure is
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19 coloured blue. The Zn^{2+} ion is shown as a grey sphere.
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Table 1: Conservation of enzymes that modify lipid A (modified from Miller et al.¹⁹)

Species	PhoPQ	PmrAB	EptA*	ArnT	LpxO	PagP	PagL
<i>Salmonella</i> ^{42 54, 55, 102}	+	+	+	+	+	+	+
<i>Shigella</i> ^{112, 113}	+			+	-	+	-
<i>Yersinia</i> ¹¹⁴	+			+	-	+	-
<i>E. coli</i> ^{102, 114-116}	+	+	+	+		+	-
<i>Pseudomonas</i> ^{117, 118}	+	+		+	+	+	+
<i>Bordetella</i> ^{50, 119}	+			+	+	+	-
<i>Legionella</i> ^{50, 120}						+	
<i>Neisseria</i> ^{53, 77, 121}	+		+	-	-	-	-
<i>Erwinia</i> ¹²²	+			+	-	+	-
<i>Brucella</i> ¹⁹	+			-	+	-	-
<i>Camphylobacter</i> ^{75, 81, 114}			+				
<i>Klebsiella pneumoniae</i> ^{115, 123}	+	+		+		+	
<i>Yersinia pestis</i> ^{50, 124}		+					
<i>Citrobacter rodentium</i> ^{115, 125}	+	+					
<i>Vibrio cholerae</i> ¹¹⁴						+	
<i>Proteus mirabilis</i> ¹²⁶				+			
<i>Helicobacter pylori</i> ^{52, 114, 127}	+		+				

*The name EptA is that of *Neisseria*; the enzyme corresponds to PEA transferase specific to Lipid A.
+ highly conserved
- not present

(A) Representation of the cell envelope of Gram-negative bacteria showing the inner membrane, the outer membrane and the periplasmic space containing the peptidoglycan layer. The constituent molecules found in the different structures of the cell envelope are shown. (B) Architecture of LPS and LOS in Gram-negative bacteria. The sugar molecules are represented by hexagons with GlcN: N-acetylglucosamine, KDO: 3-deoxy-D-manno-octulosonic acid (keto-deoxyoctulosonate), Hep: heptose, Gal: galactose and Glu: glucose. Phosphates are represented by a green circle labelled P.

250x287mm (300 x 300 DPI)

Schematic representation of the activation and interaction of PhoPQ and PmrAB two component system leading to enhanced production lipid A modifying enzymes. P denotes phosphate.

101x88mm (300 x 300 DPI)

(A) Chemical structure of Lipid A showing the enzymes that carry out modifications to the lipid substrate and the sites where modifications occur. (B) Schematic overview of innate immune signaling by LPS/LOS leading to production of CAMPs, cytokines and co-stimulatory molecules.

101x99mm (300 x 300 DPI)

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365x363mm (300 x 300 DPI)

Secondary structure representation for PagL (PDB: 2ERV). The N terminus is coloured blue and the C terminus is coloured red. The strands making up the β -barrel are labelled as A to H. Extracellular loops are labelled as L and periplasmic facing turns are labelled as T. Proposed active site residues (Glu140, His 126 and Ser128) are shown in magenta stick representation. The membrane surface is depicted as a grey line and the residues comprising the aromatic belts at the membrane interfaces are shown in grey stick representation.

229x187mm (300 x 300 DPI)

(A) Secondary structure representation of EptA from *Neisseria meningitidis* (PDB: 5FGN). The N terminus is coloured blue and the C terminus is coloured red. The two domains are labelled as TM for transmembrane domain and SD for soluble domain. The bridging helix is labelled as BH and the transmembrane helices are labelled and numbered as TMH1 through TMH5. The membrane surface is depicted as a grey line and the residues comprising the aromatic belts at the membrane interfaces are shown in grey sticks. The bound DDM molecules is shown in ball and stick representation. Inset depicts a close up of the active site with the side chains included for residues that coordinate the Zn²⁺ atom (Glu240, Asp452 and His453) as well as the proposed nucleophile, Thr280 shown in white stick representation. (B) Close up of the soluble domain of EptA showing the positions of the disulphide bonds as yellow sticks. (C) Close up of the proposed substrate binding region of EptA. The periplasmic facing helices, PH2 and PH2', are colored in light blue. The bound DDM is shown in ball and stick representation. The Zn²⁺ ion is shown as a grey sphere.

338x325mm (300 x 300 DPI)

(A) Secondary structure representation of ArnT from *Cupriavidus metallidur* (PDB:5EZM) in the apo form and (B) in the UndP bound form (PDB: 5F15). The N termini are coloured blue and the C termini coloured red. The two domains are labelled as TM for transmembrane domain and SD for soluble domain. The transmembrane helices are labelled and numbered as TMH1 through TMH12. The periplasmic helix, PH3, is indicated as is the periplasmic facing loop PL4. The membrane surface is depicted as a grey line and the residues comprising the aromatic belts at the membrane interfaces are shown in grey sticks. Inset of panel A shows a close up of the Zn²⁺ binding site with the metal shown as a grey sphere, and coordinating residues (Glu84, His265 and His267) represented as white sticks. Inset of panel B shows the close up of the UndP binding site with side chains of residues (Lys85, Arg278 and Tyr345) implicated in ligand binding shown in white sticks. (C) Superposition of the apo and UndP bound ArnT structures. The apo structure is coloured pink and the UndP-bound structure is coloured blue. The Zn²⁺ ion is shown as a grey sphere.

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