

A hydrocarbon ruler measures palmitate in the enzymatic acylation of endotoxin

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The ability of enzymes to distinguish between fatty acyl groups can involve molecular measuring devices termed hydrocarbon rulers, but the molecular basis for acyl-chain recognition in any membrane-bound enzyme remains to be defined. PagP is an outer membrane acyltransferase that helps pathogenic bacteria to evade the host immune response by transferring a palmitate chain from a phospholipid to lipid A (endotoxin). PagP can distinguish lipid acyl chains that differ by a single methylene unit, indicating that the enzyme possesses a remarkably precise hydrocarbon ruler. We present the 1.9 Å crystal structure of PagP, an eight-stranded β-barrel with an unexpected interior hydrophobic pocket that is occupied by a single detergent molecule. The buried detergent is oriented normal to the presumed plane of the membrane, whereas the PagP β-barrel axis is tilted by approximately 25°. Acyl group specificity is modulated by mutation of Gly88 lining the bottom of the hydrophobic pocket, thus confirming the hydrocarbon ruler mechanism for palmitate recognition. A striking structural similarity between PagP and the lipocalins suggests an evolutionary link between these proteins.

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Introduction

The assembly and maintenance of biological membranes depend critically on the specificity of lipid acyl group transfer reactions (Vance and Vance, 2002). As lipids are key components of biological membranes, many enzymes of lipid metabolism are integral membrane proteins. While significant advances have been made in the structural study for some classes of membrane proteins, only two structures are known for integral membrane enzymes of lipid metabolism. These are OMPLA and PagP, and both are β-barrel proteins from the outer membrane of *Escherichia coli*. The outer membrane of Gram-negative bacteria is an asymmetric bilayer, with a periplasmic phospholipid leaflet and an outer leaflet that is normally restricted to the lipid A (endotoxin) component of lipopolysaccharide (Kamio and Nikaido, 1976). The phospholipase OMPLA is a 12-stranded antiparallel β-barrel with its active site located at a Ca²⁺-stabilized dimeric interface (Snijder *et al*, 1999). OMPLA is thought to function in the removal of phospholipids that aberrantly migrate into the outer membrane outer leaflet (Dekker, 2000), and has little specificity for the length of the acyl chain of the lipid substrate.

In contrast, PagP is an outer membrane acyltransferase that catalyzes the transfer of a palmitate group from the *sn*-1 position of a phospholipid to the N-linked R-3-hydroxymyristate chain on the proximal glucosamine unit of lipid A (Figure 1). The simplest lipopolysaccharide structure encountered by PagP in outer membranes is lipid A, a phosphorylated and acylated disaccharide of glucosamine, bearing two 3-deoxy-D-manno-2-octulosonic acid (Kdo) sugars (Raetz and Whitfield, 2002). Despite the presence of 18-carbon acyl groups in bacterial phospholipids, only 16-carbon palmitoyl groups are transferred by PagP to lipid A *in vivo* (Guo *et al*, 1998), and PagP has the unusual ability to distinguish between phospholipid acyl groups that differ by only a single carbon atom (Brozek *et al*, 1987; Bishop *et al*, 2000). The active site residues of PagP map to the extracellular surface of the outer membrane, indicating that phospholipids must first migrate into the outer leaflet before the reaction can proceed (Hwang *et al*, 2002).

The product of the PagP reaction is palmitoylated lipid A, which provides resistance to vertebrate antimicrobial peptides (Guo *et al*, 1998) and antagonizes endotoxin signaling through the human toll-like receptor 4 pathway (Tanamoto and Azumi, 2000; Muroi *et al*, 2002; Kawasaki *et al*, 2004). PagP has been shown to promote infections of the mammalian respiratory tract by providing bacterial resistance to host immune defenses (Robey *et al*, 2001; Preston *et al*, 2003), including antibody-mediated complement lysis (Piloni *et al*, 2004). The *pagP* gene is regulated by virulence-associated signal transduction pathways including *Salmonella* PhoP/PhoQ (Guo *et al*, 1998) and *Bordetella* BvgA/BvgS (Preston *et al*, 2003), and lipid A palmitoylation appears to be a regulated process in other pathogens of humans

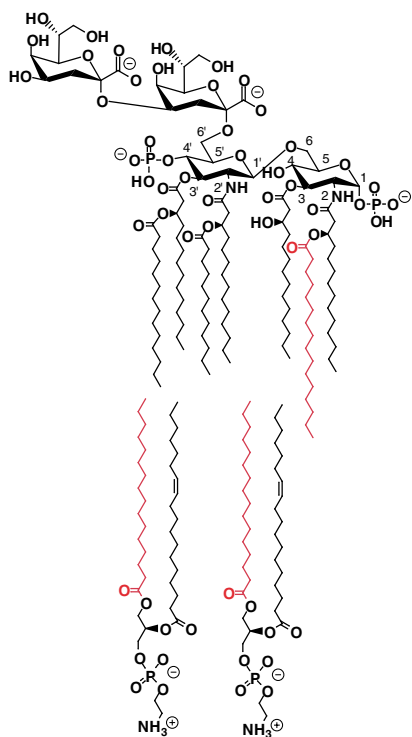


Figure 1 The asymmetric outer membrane of Gram-negative bacteria consists of an inner phospholipid leaflet (indicated by the two molecules of phosphatidylethanolamine in the lower part of the panel) and an outward-facing leaflet of lipid A bearing minimally two 3-deoxy-D-manno-2-octulosonic acid (Kdo) sugars (indicated by a Kdo₂-lipid A in the upper part of the panel, shown as the palmitoylated product of the PagP reaction). PagP catalyzes the transfer of a palmitate chain (shown in red) from the *sn*-1 position of a phospholipid donor to the lipid A acceptor. This transfer requires the migration of the phospholipid donor to the outer leaflet of the outer membrane, presumably by mechanisms that do not directly involve PagP.

(Rebeil *et al*, 2004), insects (Derzelle *et al*, 2004), and plants (Fukuoka *et al*, 2001).

The ability of enzymes to distinguish between fatty acyl groups with varying numbers of carbon atoms may involve molecular measuring devices termed 'hydrocarbon rulers' (Wyckoff *et al*, 1998). Hydrocarbon rulers have been described in the structures of certain soluble enzymes (Tarshis *et al*, 1996; Long *et al*, 1998; Wyckoff *et al*, 1998); however, the molecular basis for acyl-chain recognition in any membrane-bound enzyme remains to be defined. The global fold and dynamics of *E. coli* PagP have been determined by NMR spectroscopy, and revealed an eight-stranded antiparallel β -barrel preceded by an N-terminal amphipathic α -helix (Hwang *et al*, 2002). Key residues for catalysis mapped to a highly dynamic cell surface loop, but neither the position of the amino-acid side chains nor the presence of any bound ligands or detergents was determined in the NMR structure. Here we describe the crystal structure of PagP at 1.9 Å resolution, which unexpectedly reveals a bound detergent molecule in the β -barrel interior. Based on this observation, we determined the detergent inhibition properties of the enzyme, and used site-directed mutagenesis experiments to verify the role of the interior detergent-binding site in acyl-chain recognition. These studies provide the first molecular description of a hydrocarbon ruler in an integral membrane enzyme.

Results and discussion

Expression and purification of PagP

We were able to crystallize the purified protein either solubilized from native *E. coli* membranes or refolded from inclusion bodies. Natively folded protein was purified from membranes via selective solubilization with the detergent lauryldimethylamine oxide (LDAO), followed by NiNTA chromatography, as previously described (Bishop *et al*, 2000). For the refolded protein, PagP was targeted to insoluble aggregates by expressing the protein without its native leader sequence (Hwang *et al*, 2002). Inclusion bodies were isolated and solubilized in GuHCl, and the protein was refolded by fast dilution into solutions of 0.5% LDAO and further purified by ion-exchange chromatography. The yield of refolded protein was typically greater than 50%. Both the natively folded and refolded forms of the protein exhibited similar temperature-dependent shifts in electrophoretic mobility by SDS-PAGE, displayed indistinguishable specific activities, and crystallized under similar conditions in the same space group with comparable unit cells. We continued the structural studies with the refolded protein because this material produced larger and more reproducible crystals.

Structure and organization of PagP in outer membranes

We determined the crystal structure of *E. coli* PagP to 1.9 Å resolution. As expected, PagP is an eight-stranded antiparallel β -barrel with a periplasmic N-terminal amphipathic α -helix (Table I and Figure 2A). The average C α r.m.s.d. with the average NMR backbone structure (Hwang *et al*, 2002) is 1.8 Å, excluding the leading α -helix and all connecting loops (Figure 3A). The enzyme has a barrel shear number of 10 and is thus topologically similar to OmpA (Pautsch and Schulz, 2000) and NspA (Vandeputte-Rutten *et al*, 2003), although the PagP barrel is considerably shorter (Figure 2). The barrel cross-section is approximately circular. Consistent with previous structures of β -barrel outer membrane proteins, short loops are found facing the periplasmic surface, while larger, more flexible loops face the cell exterior (Koebnik *et al*, 2000). In all, 10 residues from the first outward-facing loop L1 (Tyr38–Asn47) are not visible in the electron density maps, and this loop displayed highly variable conformations in the NMR ensemble of structures (Hwang *et al*, 2002; Figure 3A). None of the intermolecular contacts in the crystal lattice suggest that PagP is a dimer or higher order oligomer, and we assume that the protein is monomeric in its native state.

While all previously described outer membrane proteins are oriented in the membrane with their β -barrel axis normal to the plane of the bilayer (Wimley, 2002), a representation of the protein colored according to side-chain hydrophobicity clearly revealed a nonpolar band of appropriate width to span the membrane, but this band was not aligned with the barrel axis. A quantitative analysis of the distribution of the exposed hydrophobic/hydrophilic surfaces of the protein indicates a 25° tilt of the PagP barrel axis with respect to the membrane normal (Figure 3). The hydrophobicity profile of the untilted protein is not consistent with that seen in other membrane proteins (Wimley, 2002), since the middle hydrophobic region is too shallow and there is no clear and sharp difference between the central membrane-embedded and the flanking solvent-exposed regions. In the tilted position, however, the

Table 1 Crystallographic statistics

Diffraction data	Native	NaBr	CsCl	SeMet
Resolution (Å)	1.9	2.7	2.6	2.6
Unit cell (Å)				
a,b	50.67	51.02	51.12	50.60
c	158.44	158.31	158.23	158.06
Wavelength (Å)	0.9505	0.9050	0.9505	1.54
Reflections collected	227 607	48 970	71 093	49 340
Unique reflections	17 041	5967	6925	6217
Completeness (%)	99.5 (99.9)	93.9 (94.5)	99.0 (100.0)	89.8 (93.4)
$\langle I \rangle / \langle \sigma I \rangle$	38.1 (9.0)	17.9 (5.6)	21.2 (7.7)	29.6 (4.2)
R_{sym} (%)	5.0 (33.7)	8.6 (45.2)	7.8 (38.5)	6.2 (39.8)
<hr/>				
Refinement	Native			
Resolution (Å)	20.0–1.90			
Data cutoff $F/\sigma(F)$	0			
R/R_{free} , 5% (%)	21.65/25.55			
R.m.s.d. bond lengths (Å)	0.015			
R.m.s.d. bond angles (deg)	1.44			
Number of atoms/residues				
PagP	1225/157			
LDAO	66/3 complete, 2 partial			
Acetate	20/3			
MPD	16/2			
Waters	38			

Numbers in parentheses refer to the highest resolution shell.

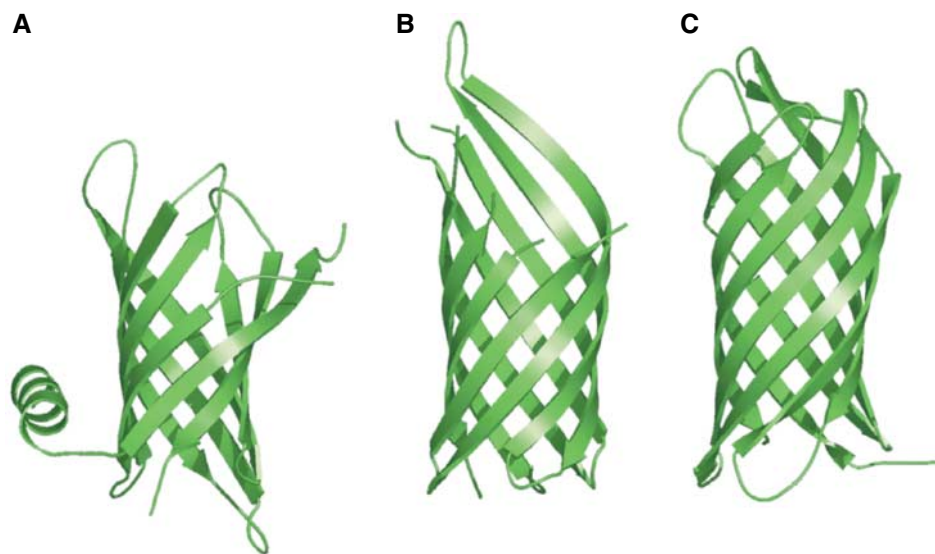


Figure 2 Ribbon diagrams of (A) PagP, (B) OmpA (Protein Data Bank code 1QJP; Pautsch and Schulz, 2000), and (C) NspA (Protein Data Bank code 1P4T; Vandeputte-Rutten *et al*, 2003).

protein surface shows the expected hydrophobic zone of appropriate width (roughly 24 Å), with sharp transitions on either side to strongly hydrophilic regions (Figure 3C). A tilted barrel is also supported by the positions of aromatic belt residues (Schulz, 2002), which cluster strongly at the membrane interface regions only when the barrel is in this position (Figures 3 and 4). The protein in this orientation presents a relatively flat surface on either side of the membrane, without prominent protruberances. The spaces caused by the tilting of the main barrel are filled with the N-terminal amphipathic α -helix on the periplasmic side, and a β -bulge at Pro28 near the end of strand A on the opposite extracellular side (lower right and upper left of Figure 3B, respectively).

The amphipathic helix lies along the lipid/water interface, with a polar face exposed to the periplasmic space, and the opposite face interacting with the barrel and the inner phospholipid leaflet of the outer membrane. This helix contacts the periplasmic ends of strands B and C, which do not have aromatic belt residues, and contributes the conserved W17 to the belt. Although the helix was not positioned relative to the rest of the protein in the NMR structure (Figure 3A), we expect that the orientation of the helix relative to the barrel in the crystal structure represents a true contact that occurs in bilayers.

The tilt angles for each strand relative to the barrel axis range from 38 to 42°, as expected for an eight-stranded barrel

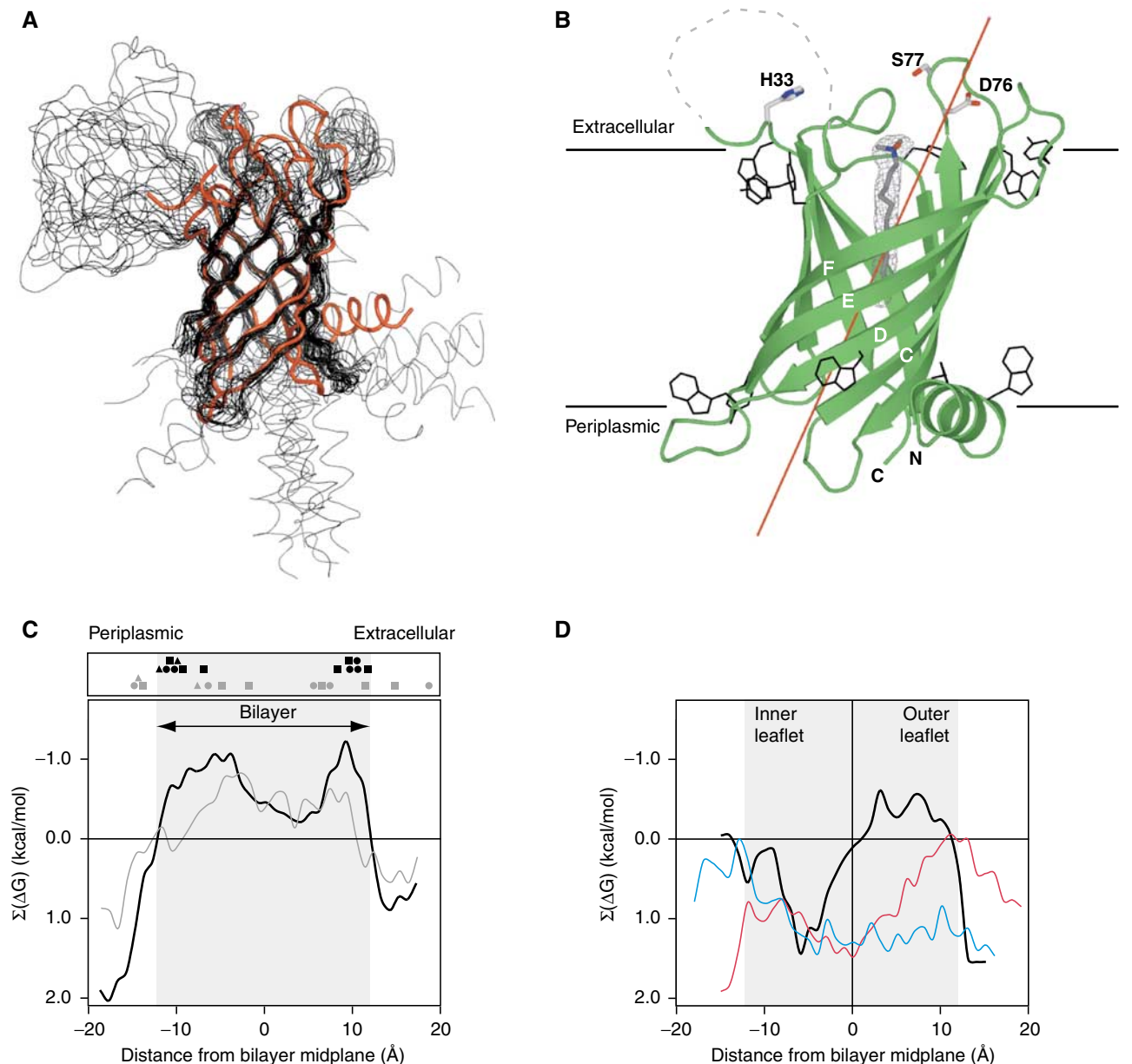


Figure 3 (A) Superposition of the PagP crystal structure (red) with the 20 lowest energy NMR structures in DPC micelles (black) (Hwang *et al*, 2002; Protein Data Bank code 1MM4). The leading α -helix is at the lower right, and loop L1 is at the upper left. (B) Ribbon diagram of PagP. $2||F_o|-|F_c||$ electron density for the internal LDAO ligand is shown in gray mesh, and the aromatic residues located at the presumed membrane/water interfaces are shown in black. Residues 38–47 of extracellular loop L1 are not visible in the electron density maps and are indicated by the dashed segment. The barrel axis is tilted approximately 25° from the membrane normal and is shown as a red line. The horizontal lines represent the presumed position of the outer membrane bilayer. (C) Hydrophobicity profiles (Wimley, 2002) for the outward-facing PagP residues as a function of membrane position are shown as solid lines. Negative $\Sigma(\Delta G)$ values indicate regions that are more hydrophobic. The gray line and symbols present results for the protein positioned with the β -barrel axis aligned along the membrane normal, while the black line and symbols are for the protein tilted as in panel B. The solid symbols represent the C_γ positions of the Trp (squares), Tyr (circles), and Phe (triangles) residues that form the inner (Trp17, Tyr23, Trp89, Trp93, Phe101, Tyr133, Phe161) and outer (Trp32, Trp51, Trp81, Tyr119, Tyr142, Tyr153) aromatic belts. (D) The hydrophobicity profiles for the inward-facing residues of PagP (black), OmpA (blue), and NspA (red) indicate an unusually hydrophobic interior in the upper half of the PagP β -barrel.

with a shear number of 10 (Schulz, 2002). However, because of the tilting of the barrel in the bilayer, there are large differences in the exposure of each of the eight strands to the lipidic environment of the membrane. Strands A, B, and H are roughly aligned with the membrane normal (angle $\sim 20^\circ$) and cross the bilayer with 6–8 residues. In contrast, strands D, E, and F are tilted by $\sim 50^\circ$ relative to the membrane

normal and require roughly 12 residues to span the bilayer (Figure 4).

The protein interior includes a binding pocket for lipid acyl chains

Relative to all previously described β -barrel outer membrane proteins, PagP has an unusual interior: the upper half of the

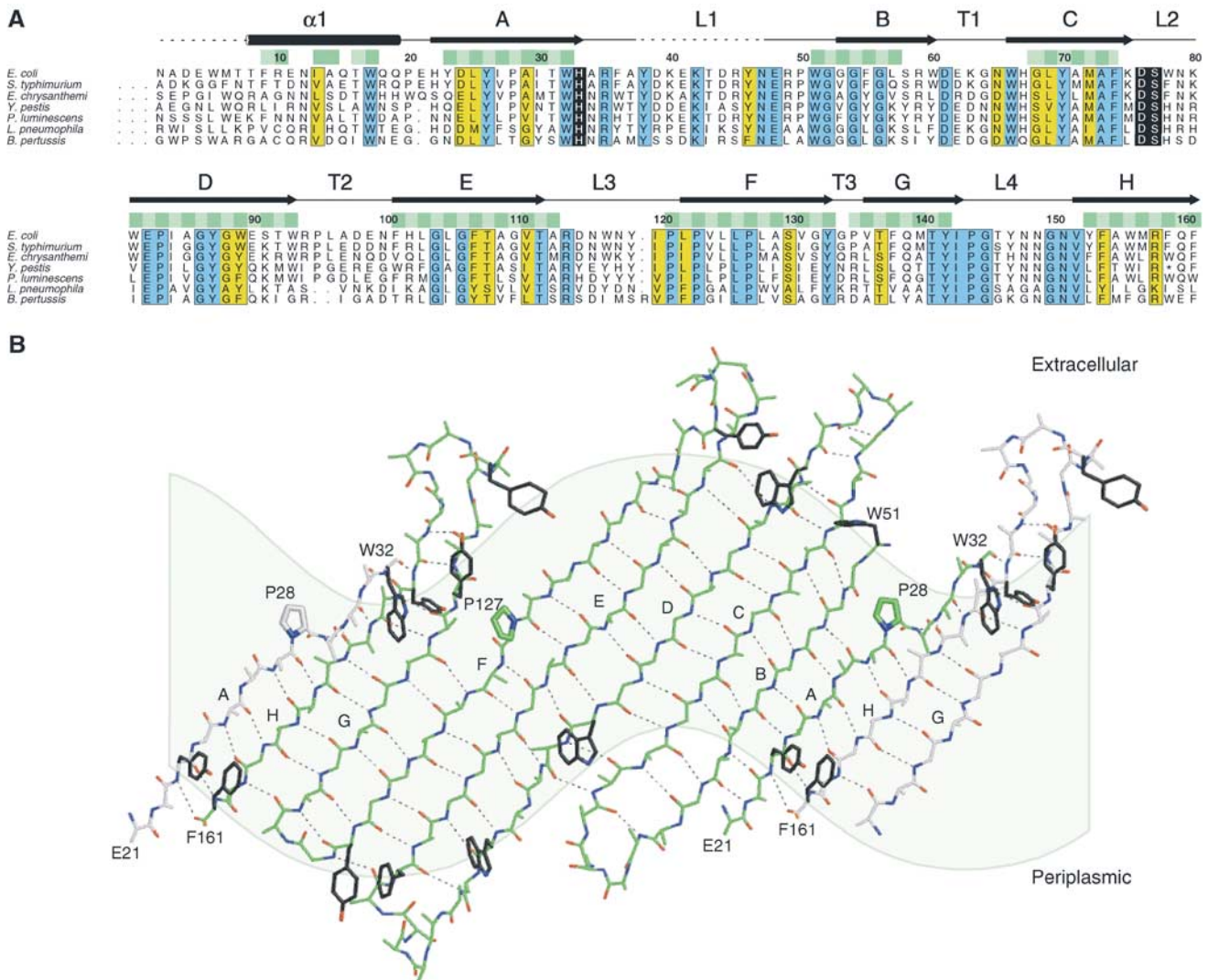


Figure 4 (A) Multiple sequence alignment of PagP homologues from *E. coli*, *Salmonella typhimurium*, *Erwinia chrysanthemi*, *Yersinia pestis*, *Photobacterium luminescens*, *Legionella pneumophila*, and *Bordetella pertussis*. To date, the enzyme has been found primarily in pathogenic Gram-negative bacteria, and a representative species from each genus is included in the alignment. Residues highlighted in blue boxes are identical in all known homologues, and conserved residues are shown in yellow boxes. The putative catalytic residues are included in black boxes. Residues that are located within the bilayer are indicated with horizontal green bars, with the lipid-exposed positions shown in darker green. The residue numbering is based on the *E. coli* PagP sequence of the mature protein without the leading signal sequence. Disordered regions in the crystal structure are indicated by the dashed lines. The N-terminal amino acid of the mature *E. coli* protein was determined by Edman degradation (Bishop *et al*, 2000). The N-terminal amino acids of the other homologues following the removal of the signal sequences have not yet been experimentally determined. (B) An ‘unrolled’ barrel was generated by projecting atomic positions onto a cylinder aligned with the barrel axis and laid flat. The light green shading indicates the position of the membrane, which follows a sinusoidal path because of the tilting of the barrel in the bilayer. One central PagP molecule is drawn with green carbon atoms, while flanking strands are shown with white carbon atoms. Residues 8–20 of the leading α -helix and residues 33–37 and 48–50 from L1 are omitted for clarity. Side chains are omitted, except for those of prolines 28 and 127 and residues from the aromatic belts. Interchain hydrogen bonds are drawn with dashed lines.

barrel core is distinctly hydrophobic and is devoid of interior waters, while the lower half has a typical hydrophilic interior filled with polar side chains (Figure 3D). These two distinct interiors map to the upper lipid A leaflet and to the lower phospholipid leaflet of the membrane, respectively.

During the course of refinement, difference electron density maps revealed the presence of several LDAO detergent molecules associated with the exterior surface of the barrel, and a single detergent molecule nested within the upper hydrophobic half of the protein core (Figure 3B). The LDAO bound inside the barrel is roughly aligned with the expected membrane normal, and is oriented with its polar head group

facing the extracellular region and its acyl tail extending into the protein center. The cavity is lined with hydrophobic residues and extends approximately halfway through the barrel (Figures 3D and 5), corresponding to the region of the protein that is embedded in the outer lipid A leaflet of the membrane. The residues that contact the bound LDAO are highly conserved in the PagP homologues (Figures 5C and 4A). The putative active site residues His33, Asp76, and Ser77 are located on the extracellular surface of the barrel with their side chains oriented towards the central axis of the barrel (Figure 3B), and several solvent molecules are found in a sequestered pocket located near these residues.

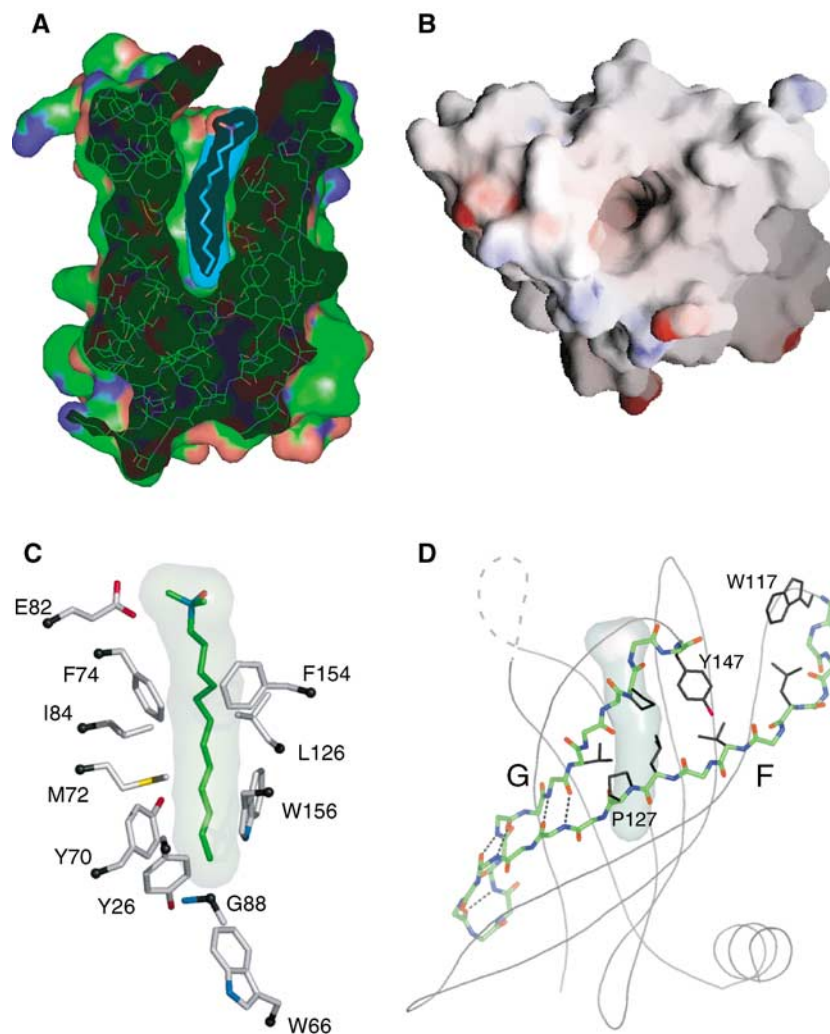


Figure 5 Ligand interactions in PagP. **(A)** Cut-away view of the protein, showing the inner ligand pocket and the internally bound detergent molecule. **(B)** Overhead view of the empty barrel in surface representation, colored according to the electrostatic surface potential. **(C)** Local environment of the internal LDAO detergent. Side-chain C α carbons are indicated in black. **(D)** Strands F and G are shown in stick representation, and form main-chain interstrand H bonds only in the bottom part of the barrel. In the upper part of the barrel, the space between the two strands is filled with side chains from apolar residues. Hydrogen bonds are indicated with dashed lines, and the side chains for residues Trp117, Leu122, Val124, Leu126, Pro127 (strand F) and Thr141, Pro144, and Tyr147 (strand G) are shown in black.

However, the deeper hydrophobic region of the pocket is completely filled with the detergent acyl chain and does not contain any interior waters.

Nine water molecules are located in the lower hydrophilic portion of the PagP interior, and this lower part of the protein has features seen in the overall interior of other outer membrane proteins, including an extensive network of hydrogen-bonding and salt-bridge interactions. The interior cores of previously solved structures of outer membrane β -barrel proteins are filled with polar side chains (Schulz, 2002; Wimley, 2002), including eight-stranded members of the family and members with larger barrels, such as the porins. An interesting new example is the NalP autotransporter β -domain, a 12-stranded barrel with a highly charged interior that is filled with an α -helical peptide from the N-terminus of the protein (Oomen *et al*, 2004).

Some vague similarities to the PagP structure are seen in the outer membrane protein NspA, which has a hydrophilic interior within the transmembrane domain and a

bound detergent molecule located at the top of its barrel (Vandeputte-Rutten *et al*, 2003). However, in this case, the detergent lies across a shallow groove at a position external to the bilayer and remains largely exposed to the solvent. The limited depth of this binding site can be seen in the analysis of the hydrophobicity of inward-facing residues in NspA (Figure 3D).

A hydrocarbon ruler determines palmitate specificity

Based on these structural data, several predictions can be made regarding the mechanism of action of PagP. PagP has a typical polar core only in the lower half of the protein, and a distinctly hydrophobic interior in the region that would be located in the outer lipid A leaflet of the membrane bilayer (Figure 3D). We believe that the LDAO molecule bound in the hydrophobic pocket occupies the palmitate transfer binding site, and hypothesize that internalization of a phospholipid acyl chain occurs during the transfer reaction.

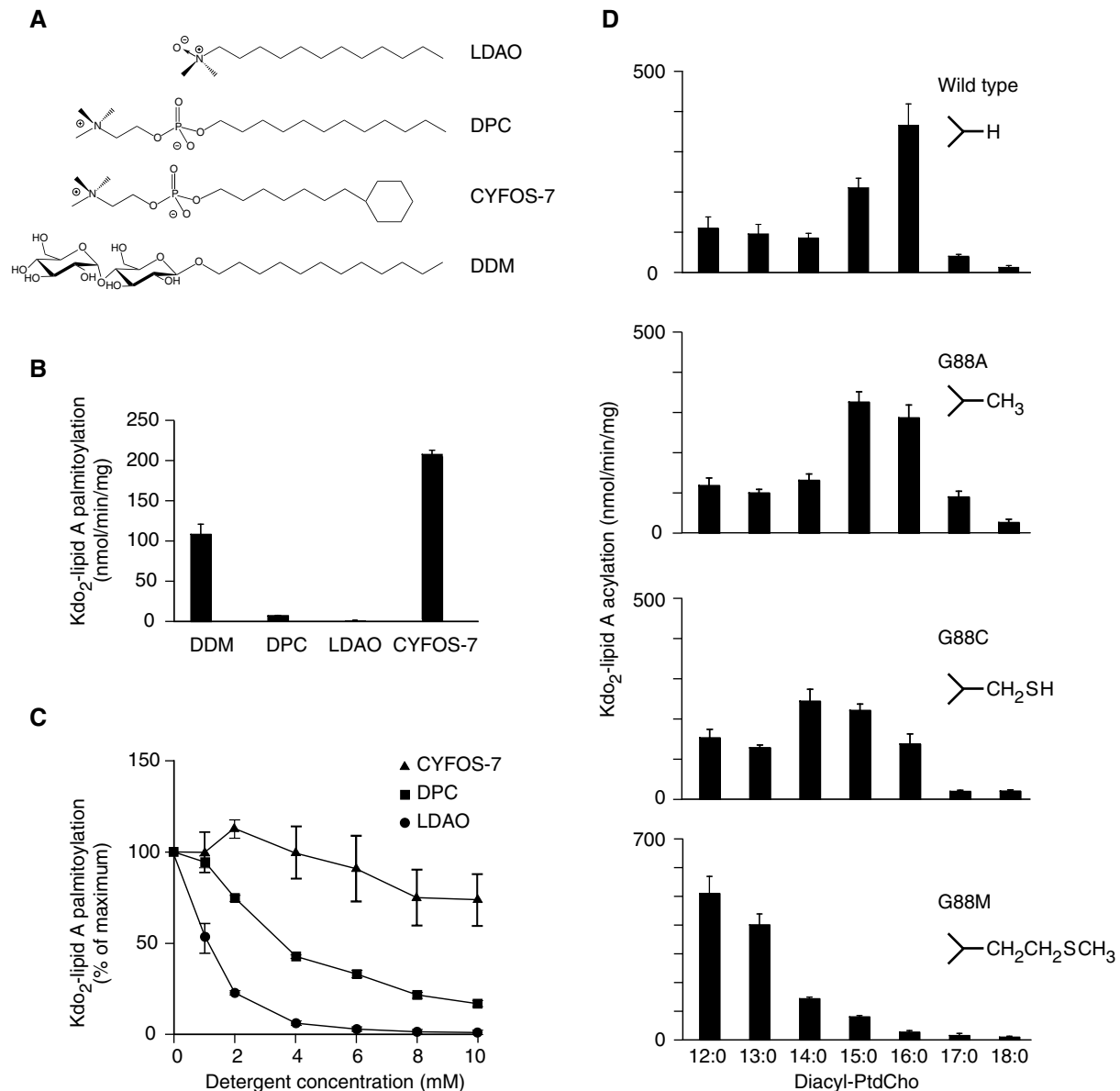


Figure 6 Enzymatic activity of PagP. (A) LDAO and DPC are relatively compact amphiphiles, while CYFOS-7 and DDM have bulky tail and head groups, respectively. (B) PagP is active in CYFOS-7 and DDM, but shows little activity in DPC or LDAO. (C) PagP in 0.25% DDM is inhibited by DPC and LDAO. (D) The PagP donor acyl-chain specificity in phosphatidylcholine (PtdCho) is a function of the amino-acid side chain at position 88. The wild-type protein has Gly88 and preferentially transfers a palmitate group to Kdo₂-lipid A. The G88A, G88C, and G88M mutants provide side chains that occupy the acyl-binding pocket of PagP to progressively greater extents, and decrease the specificity of the transferred chain by 1, 2, and 4 methylene equivalents, respectively.

The relevance of the detergent-binding site in PagP is confirmed by data in which LDAO and dodecylphosphocholine (DPC) act as inhibitors of PagP transferase activity, while detergents such as dodecyl- β -D-maltoside (DDM) and CYFOS-7 that cannot fit in the barrel core have no inhibitory effect (Figure 6A–C). The hydrophobic tail of CYFOS-7 terminates with a cyclohexyl group and would not fit into the PagP acyl cavity, while the disaccharide headgroup of DDM would exclude it from the binding site. Molecular modeling suggests that a C16 ligand in place of the shorter LDAO molecule would position the headgroup in proximity to the catalytic Asp76/Ser77 residues.

To further assess the biological significance of the hydrophobic pocket, we characterized a series of mutants

designed to test the idea that the dimensions of the pocket determine the specificity of the length of the transferred acyl chain. Gly88 is located on strand D near the center of the barrel and forms part of the ‘floor’ of the inner cavity. The pro-S hydrogen on the C α of this residue is oriented directly towards the terminal methyl group of the bound detergent (Figure 5C). A series of mutations, G88A, G88C, and G88M, were tested for substrate specificity (Figure 6D). We observed that PagP mutants with longer amino-acid side chains at position 88 preferentially transferred shorter acyl chains in donor phosphatidylcholines. In effect, a ‘hydrocarbon ruler’ allows PagP to discriminate between linear acyl groups with different numbers of carbon atoms.

Determinants of lipid trafficking to the active site

The lipid A acceptor in the PagP transfer reaction is anchored in the outer leaflet of the outer membrane, but the phospholipid donor is normally restricted to the inner leaflet (Kamio and Nikaido, 1976). We presume that translocation of a phospholipid into the outer leaflet is followed by internalization of the *sn*-1 acyl chain into the PagP interior. The route of entry for a hydrophobic ligand into the PagP interior poses an interesting structural question, since an approach along the long axis of the ligand-binding pocket is not an option here, as it would be with a soluble protein. Instead, the internalization most likely occurs via a lateral route from the membrane phase to the interior of the protein, and the absence of hydrogen bonding between strands A/B and F/G in the upper half of the membrane-embedded region provides two plausible gateways for access of lipid substrates to the β -barrel interior (Figure 4B). The main-chain interactions between strands F and G are disrupted in part by residue Pro127, which is conserved in all known PagP homologues (Figure 4A). In this region, the wall of the barrel is made from nonpolar side-chain–side-chain interactions (Figure 5D), and side-chain reorientations may provide an energetically reasonable pathway for entry and/or exit of ligands to the binding pocket. A second site that may also play a role in providing access to the active site is localized near the β -bulge residue Pro28 on strand A. This residue interrupts the hydrogen bonding with strand B, and causes a change in registration of the H-bonding pattern between strands A and H. This region involves the highly dynamic loop L1 connecting strands A and B (Hwang *et al*, 2002), and recent NMR studies of PagP dynamics in the noninhibitory detergent CYFOS-7 have revealed a conformational change in the L1 loop and adjacent regions of the β -barrel (Hwang *et al*, 2004). A multiple sequence alignment of PagP homologues (Figure 4A) shows that the L1 region contains several highly conserved residues and does not contain any insertions or deletions, further supporting an important functional and structural role for this part of the protein.

The requirement of invariant His33 in L1, and Asp76 and Ser77 in L2 for catalysis (Hwang *et al*, 2002), might suggest that PagP utilizes an acyl-enzyme mechanism characteristic of known serine esterases. However, these putative active site residues are not organized into a catalytic triad in the crystal structure (Figure 3B), nor in the NMR structure in DPC (Figure 3A; Hwang *et al*, 2002). Given the presence of two possible sites for access of lipid substrates to the β -barrel interior (Figure 4B), the PagP structure is also compatible with a ternary complex mechanism for direct transfer of the palmitoyl group from the donor phospholipid to the endotoxin acceptor. Either mechanism requires a stereospecific interaction with lipopolysaccharide, and PagP contains several highly conserved arginine and lysine residues in the exterior-facing loops L1–L3 (Figure 4A). A structural motif for lipopolysaccharide recognition involving four positively charged amino acids has been identified in lipopolysaccharide-interacting proteins (Ferguson *et al*, 2000). We expect some variability in the recognition motifs in the PagP family of sequences because there are known variations in both lipopolysaccharide structure and PagP stereoselectivity in different bacterial species. However, the invariant Lys42 in the disordered region of the L1 loop has been mutated to an Ala with the observation that this mutant retains a slow

phospholipase activity that is found in the wild-type enzyme, but is defective in its ability to palmitoylate lipid A. This observation is consistent with a role of Lys42 in lipopolysaccharide recognition (EI Lo and RE Bishop, unpublished observations). Clearly, detailed knowledge of the structural transitions that occur in the L1 loop and the surrounding regions (Hwang *et al*, 2004) will hold the key to elucidating the nature of the interactions of PagP with phospholipids and lipopolysaccharides, and we expect that this insight will ultimately lead to an understanding of the catalytic mechanism.

Structural relationships with the lipocalins

A striking similarity is seen between the structures of PagP and the lipocalins, a widely distributed family of generally soluble lipid-binding proteins, the archetype of which is serum retinol-binding protein (RBP) (Newcomer and Ong, 2000). Both PagP and RBP are eight-stranded antiparallel β -barrels with a deep lipid-binding pocket located inside one end of the barrel and a flanking α -helix at the other end (Figure 7). The structures superpose with the proper alignment of the β -strands (i.e. A–H of PagP superposes with A–H of RBP) with an r.m.s.d. of 2.9 Å over 93 C α atoms. The structural similarity between OmpA and the lipocalins has been noted (Pautsch and Schulz, 2000), but there are significant differences in the length of the barrels (Figure 2A and B) and in the nature of the protein interiors. The case for homology is strengthened in the case presented here because the PagP and RBP barrels have a higher percentage of alignable positions, and the two proteins have similar inner lipid-binding pockets (Figure 7). Furthermore, in the lipocalins, a long loop spans strands A and B and typically forms a lid that partially closes the ligand-binding site (Flower *et al*, 2000). It is intriguing that the long L1 loop from PagP is at an equivalent position and is strongly implicated in the function

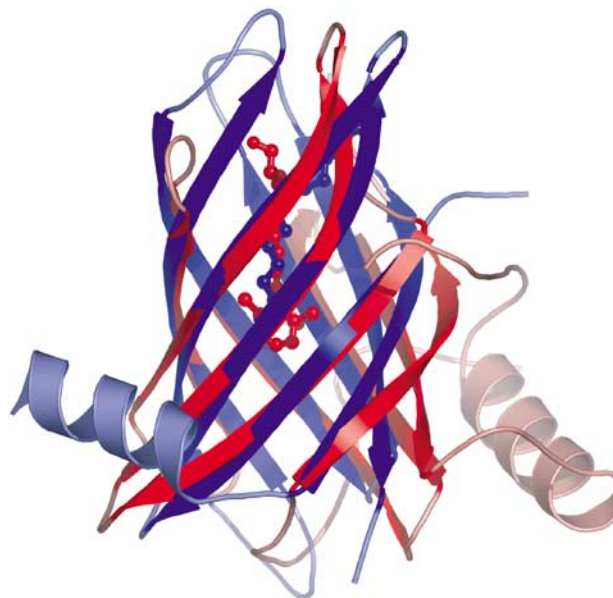


Figure 7 Superposition of the PagP (blue) and retinol-binding protein (red, Protein Data Bank code 1AQB) structures. The LDAO (blue) and retinol (red) ligands are shown in ball-and-stick representation. PagP has an N-terminal α -helix, while most lipocalin structures, including RBP, have a single α -helix at the C-terminus.

of this enzyme (Hwang *et al*, 2002, 2004); however, additional structural data will be required to test whether this loop interacts directly with ligand and/or the upper region of the barrel.

We cannot rule out the possibility that this structural similarity represents a case of convergent evolution. However, based on the absence of lipocalins from those bacteria that also lack β -barrel membrane proteins (Bishop, 2000; Ganfornina *et al*, 2000), which include all non-mycolic acid-producing Gram-positive bacteria and the Archaea (Nikaido, 2003), we cannot currently exclude the hypothesis that PagP and lipocalins share a common ancestor. This hypothesis is supported by the observation that the vast majority of bacterial lipocalins are anchored in the outer membrane as lipoproteins (Bishop *et al*, 1995; Campanacci *et al*, 2004), which could represent an intermediate state in the adaptation between membrane-bound and soluble globular domains (Bishop and Weiner, 1996). Outer membrane β -barrel domains should be suitably adapted to occupy both soluble and membrane-bound conformations because both environments are encountered during the outer membrane assembly process (Voulhoux *et al*, 2003).

Conclusions

PagP is distinguished from other known β -barrel membrane protein structures by the presence of an internal ligand-binding pocket that serves as a hydrocarbon ruler. Other unusual features include a tilting of the barrel axis with respect to the plane of the membrane, and the presence of non-hydrogen-bonded regions between β -strands in the outer leaflet half of the protein that may provide routes for lateral access of lipid substrates to the interior pocket.

Many of these features are also found in the lipocalins, and the lipocalin fold provides a rich scaffold for the design of ligand-binding specificity (Skerra, 2000). As the length of the secondary acyl groups in lipid A analogues is critical for signal transduction through the toll-like receptor 4 pathway (Stover *et al*, 2004), our finding that the specificity of the PagP hydrocarbon ruler can be altered may provide enzymatic routes for the synthesis of novel endotoxin antagonists. Efforts to develop inhibitors of PagP-catalyzed lipid A palmitoylation may provide novel treatments for bacterial infections.

Materials and methods

Cloning, expression, and purification of PagP

The cloning of the *E. coli pagP* gene with a deleted leader sequence and a C-terminal His tag was described previously (Hwang *et al*, 2002). The protein was expressed in *E. coli* BL21(DE3) cells (Novagen) with isopropyl-1-thio- β -D-galactopyranoside induction (0.2 mM). Cells were harvested, resuspended in 10 mM Tris-HCl (pH 8.0) and ruptured by cavitation in EmulsiFlex-05 (Avestin). Insoluble protein was collected by centrifugation at 26 000 g, and the pellet was resuspended and washed twice with 2% Triton X-100. The protein was solubilized in 6 M GuHCl at a concentration of 5 mg/ml, and rapidly diluted 10-fold into a solution of 0.5% LDAO, and 10 mM Tris-HCl (pH 8.0) at room temperature and stirred for 4 h. The refolded protein was applied to a Poros HQ anion-exchange column in 0.05% LDAO and the bound protein was eluted with a linear gradient of elution buffer (2 M NaCl, 10 mM Tris (pH 8.0)). *E. coli* B834(DE3) grown in the presence of seleno-L-methionine was used for the expression of SeMet-substituted protein, and protein purification proceeded as with the native protein, except that 1 mM β -mercaptoethanol was included in all buffers.

Crystallization and crystallographic data collection and processing

We were able to produce small crystals of recombinant *E. coli* PagP that was purified directly from bacterial membranes, as described in Bishop *et al* (2000). These crystals formed in space group P4₁22 with unit cell dimensions $a = 50.46 \text{ \AA}$, $b = 50.46 \text{ \AA}$, and $c = 158.18 \text{ \AA}$. However, in this study, we used isomorphous crystals of significantly higher quality that were obtained from protein expressed without the signal peptide and refolded from intracellular inclusion bodies as described above. Crystals were grown in hanging drops by mixing equal volumes of 10 mg/ml protein solution with reservoir solution (30% 2-methyl-2,4-pentandiol, 0.1 M sodium citrate (pH 5.6), 0.2 M ammonium acetate). All diffraction data collections were carried out at 100 K. For the bromine and cesium derivatives, crystals were soaked briefly in mother liquor containing either 1 M NaBr or 1 M CsCl and flash frozen. Diffraction data were collected for the native and derivative crystals at beamline F2 at the Cornell High Energy Synchrotron Source, and processed with MOSFLM and SCALA (CCP4, 1994). Crystals belonged to space group P4₁22 and contained a single PagP molecule per asymmetric unit. Experimental maps derived from three wavelength bromide MAD data were not interpretable, even when supplemented with the CsCl data. We could not obtain crystals of the pure selenomethionine protein, and attempts at seeding with native protein crystals failed. However, crystals could be obtained by mixing 60% native and 40% selenomethionine protein, and we collected additional diffraction data on these crystals on a rotating anode X-ray source (CuK α). Multiple isomorphous replacement with anomalous scattering (MIRAS) phases were calculated from the NaBr, CsCl, and SeMet derivative data sets with SOLVE/RESOLVE (Terwilliger and Berendzen, 1999), and the resulting electron density maps were interpretable and could be partially traced with the automatic chain-building methods in RESOLVE. Additional model building was performed with O (Jones *et al*, 1991), and refinement and addition of water molecules were carried out with CNS (Brunger *et al*, 1998) and REFMAC (Murshudov *et al*, 1997). Molecular graphics were prepared with PYMOL (DeLano, 2002). The final model consists of residues 7–37 and 48–161 of PagP, with two additional non-native C-terminal residues, Leu162 and Glu163, resulting from the expression plasmid. The following six C-terminal histidines were not observed in the electron density maps.

Activity assays

Diacylphosphatidylcholines (diacyl-PtdChos) were obtained from Avanti Polar Lipids (Alabaster, AL) and detergents were obtained from Anatrace (Maumee, OH). ¹⁴C-sodium acetate (57 mCi/mmol) and *sn*-1,2-di-(16:0-¹⁴C)-PtdCho (100 mCi/mmol) were obtained from Perkin-Elmer Life Sciences. Kdo₂-lipid A was extracted and purified from the heptose-deficient *E. coli* strain WBB06 (Brabetz *et al*, 1997) according to Kanipes *et al* (2001), and quantified by the purpald assay (Lee and Tsai, 1999). ¹⁴C-Kdo₂-lipid A was prepared from WBB06 cells grown in 5 ml of medium containing 20 μ M (1 μ Ci/ml) ¹⁴C-sodium acetate and grown at 37°C for 5 h until OD₆₀₀ = 1.0 before isolation (Kanipes *et al*, 2001). Assays were performed by thin-layer chromatography as described previously (Bishop *et al*, 2000), but with 0.25% *n*-dodecyl- β -D-maltoside in place of Triton X-100 as the supporting detergent, unless indicated otherwise. Mutant proteins were generated with the QuikChange protocol (Stratagene) and purified from membranes (Bishop *et al*, 2000). Detergent inhibition studies utilized *sn*-1,2-di-(16:0-¹⁴C)-PtdCho at 20 μ M (4000 cpm/ μ l) and 100 μ M Kdo₂-lipid A. Hydrocarbon ruler experiments utilized ¹⁴C-Kdo₂-lipid A at 10 μ M (~110 cpm/ μ l) with *sn*-1,2-diacyl-PtdChos at 1 mM.

Coordinates

The coordinates of PagP have been deposited in the Protein Data Bank (identification code 1THQ).

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