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Lipopolysaccharide stabilizes the crystal packing of the ABC transporter MsbA

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The ABC transporter MsbA is an integral membrane protein involved in the transport of lipid A and lipopolysaccharides to the outer leaflet of the inner membrane in bacteria. Here, the critical role of the natural substrate lipopolysaccharide in the crystallization and diffraction quality of MsbA crystals is reported. Initial crystals grown in complex with ATP–vanadate alone diffracted to ~9 Å. Screening of the natural substrate lipopolysaccharides led to the crystallization of MsbA in complex with ADP–vanadate and Ra lipopolysaccharide. The increased order within the crystal lattice allowed structure determination to 4.2 Å.

1. Introduction

Integral membrane proteins represent an extensive group of macromolecules that are considerably underrepresented in the overall body of solved protein structures. Although the expression, purification and crystallization of membrane proteins each represent a significant hurdle for structure determination, another factor is the preservation of a physiologically relevant state. Since membrane proteins are embedded in the cell membrane, the effect of lipids on the protein can be critical to the final structure (Zhang *et al.*, 2003). Indeed, lipids can play a crucial role in the overall protein folding as well as in the physiological activity of some membrane proteins, including chemical modification of substrates, signal transduction and transport (for a review, see Lee, 2004).

MsbA is a member of the ATP-binding cassette (ABC) transporter group of flippases (a subgroup that is proposed to transport lipids from the inner leaflet of the membrane to the outer leaflet) and is a highly conserved gene in Gram-negative bacteria that is essential for *Escherichia coli* viability. MsbA is required for phospholipid and lipopolysaccharide (LPS) transport to the outer membrane of bacteria (Zhou *et al.*, 1998; Putman *et al.*, 2000; Doerrler *et al.*, 2001). In addition, MsbA has been shown to share significant multi-drug resistance (MDR) substrate specificity with LmrA, an ABC transporter from *Lactococcus lactis* that can functionally substitute for human MDR1 P-glycoprotein (P-gp) in lung fibroblast cells (Reuter *et al.*, 2003; van Veen *et al.*, 1998). The close protein-sequence homology of these ABC transporters certainly suggests a common origin for the transport of hydrophobic compounds. MsbA represents a potential target for the development of antibiotics and the X-ray structure may serve as a bacterial model of human MDR ABC transporters.

Lipopolysaccharide (LPS), which comprises the outer leaflet of the outer membrane in most Gram-negative bacteria, is composed of a lipid A–phospholipid core and a variable oligosaccharide domain and helps to protect the bacterium from antibiotics and environmental stress (for a review, see Raetz & Whitfield, 2002). LPS also potently activates the TLR-4 receptor of the mammalian innate (nonclonal) immune system in response to bacterial infections and in high doses is potentially responsible for septic shock, a serious medical condition that can lead to death. The degree of ATPase activity upon the interaction of various lipid A-related moieties such as Re and Ra LPS with MsbA is related to the size of the oligosaccharide domain of the LPS (Doerrler & Raetz, 2002). Rough-chemotype LPS are mutant LPS molecules and are named according to the size of the oligo-

saccharide domain. Ra, Rb, Rc, Rd and Re correspond to the first, second, third, fourth and fifth degrees of polysaccharide chain length in order of decreasing domain size (Fig. 1*d*). To investigate the structural basis of the stimulation of ATPase activity by LPS, we crystallized MsbA from *Salmonella typhimurium* in complex with ATP, vanadate (the resulting product is a transition-state mimic with ADP and a vanadate ion acting as a γ-phosphate analog) and a number of LPS species. Here, we report the impact of LPS on the stabilization of the crystal lattice.

2. Materials and methods

2.1. Crystallization

Sample preparation, crystallization and data collection have been described previously for the MsbA–Ra LPS crystal (Reyes & Chang, 2005). In summary, MsbA from ten bacterial species including *S. typhimurium* was cloned into the pET19b expression vector, overexpressed in *E. coli* host BL21 (DE3) and extracted by agitation in the presence of 1–1.2%(*w*/*v*) detergent at 277 K. For each cloned gene, we sampled a set of six detergents for solubilization that included decyl-β-maltoside (β-DM), undecyl-β-D-maltoside (β-UDM), undecyl-α-D-maltoside (α-UDM), dodecyl-β-D-maltoside (β-DDM), dodecyl-α-D-maltoside (α-DDM) and tridecyl-β-D-maltoside (β-TDM). Extracted MsbA was purified in the presence of detergent and 10% glycerol during the purification process by nickel-chelation, ion-exchange and gel-filtration chromatography. Purified MsbA was concentrated to 10 mg ml^{-1} in a YM100 Centricon filter (Millipore) in 20 m*M* Tris–HCl pH 7.5, 100 m*M* NaCl, 0.05% β-UDM. Detergent-solubilized protein was preincubated with 10 m*M* LPS (purchased from Sigma; purified from *S. typhimurium*), 15 m*M* ATP, 20 m*M* $MgCl_2$ and 1 m*M* sodium orthovanadate. Crystals were grown using the sitting-drop method at 277 K by combining protein with precipitant in a ratio of 2:1. The precipitant solution contained 100 m*M* Tris–HCl pH 7.5–8.0, 100 m*M* NaCl, 325 m*M* sodium acetate and 18–24% PEG 400. The lipids used in additive screening were PE (phosphatidylethanolamine), DMPC (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine), DOPC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine), DOGS-NTA {1,2-dioleoyl-*sn*-glycero-3-[*N*-(5-amino-1-carboxypentyl)iminodiacetic acid]succinyl} and POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine) (all purchased from Avanti Polar Lipids).

2.2. Structure determination

All data were processed using *HKL*2000 (Otwinowski & Minor, 1997). Protein phases were determined by single anomalous scattering at the mercury L_{III} edge using 2-chloromercuri-4-nitrophenol (CMNP) as a derivative. *PHASES* (Furey & Swaminathan, 1997) was used for phasing and density modification and the protein model was built using the programs *CHAIN* (Sack, 1988) and *MOLOC*. The LPS molecule from the structure of FhuA (*E. coli* K-12 LPS) was used as a starting model for Ra LPS (Ferguson *et al.*, 2000). Crystallographic refinement using *X-PLOR* (v.3.851; Brünger, 1992) and *CNS* (v.1.1; Brünger *et al.*, 1998) resulted in a model with an *R* and R_{free} of 33 and 38%, respectively. The crystallization of MsbA with Ra LPS follows the same protocol as used for the MsbA–Ra LPS crystals. Fig. 2 was generated with *PyMOL* (DeLano, 2002).

3. Results and discussion

3.1. Lipid-dependent crystal variation

Initial efforts to crystallize MsbA with ATP–vanadate (ATP-Vi) consisted of screening ten orthologs of MsbA in six detergents each.

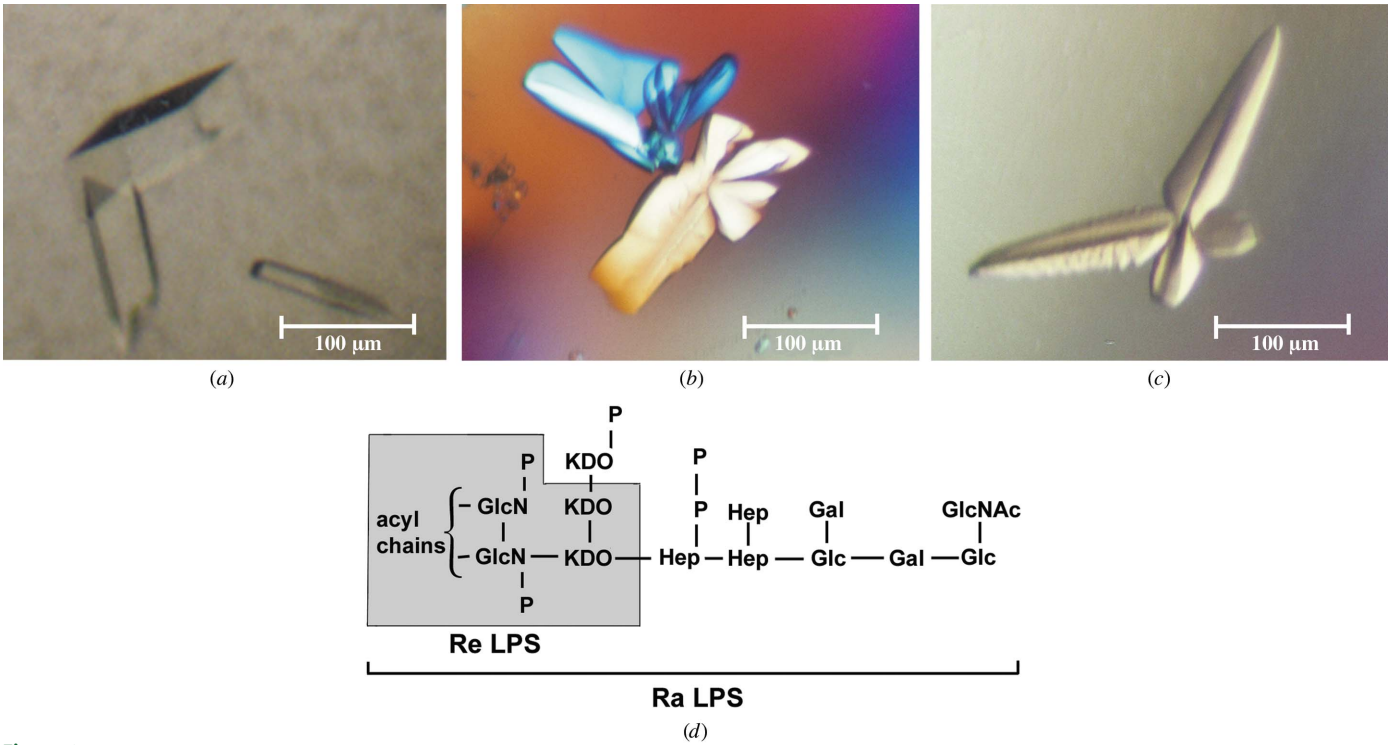

Figure 1
Crystals of (*a*) MsbA (ATP–Vi, Mg^{2+}) and crystals of the complex with (*b*) Re LPS and (*c*) Ra LPS. (*d*) Schematic diagram of the LPS structure. Shown are the Re core and the extended Ra structure. Abbreviations are as follows: KDO, 2-keto-3-deoxyoctulosonic acid; GlcN, glucosamine; Glc, glucose; GlcNac, *N*-acetyl-glucosamine; Gal, galactose; Hep, heptose; P, phosphate (not shown in this diagram nor present in our model are ethanolamine, phosphoethanolamine and aminoarabinose groups).

Each ortholog/detergent target was screened against an in-house crystallization screen. From these screens, we obtained more than 20 crystal forms, of which only two diffracted to better than 15 Å. Refinement of the crystallization conditions of the initial leads resulted in a single reproducible crystal form (Fig. 1*a*) with excellent morphology but poor diffraction (∼9 Å). Additional attempts to increase the resolution using dehydration techniques and extensive additive/lipid screening did not lead to any improvement in diffraction. We therefore took a different approach for crystallization and explored the possibility of trapping MsbA with its conjugate substrates (nucleotide and lipid).

Like most if not all MDR ABC transporters, MsbA ATPase activity is stimulated by binding of substrate (for a review, see Higgins & Linton, 2004). We altered our search of crystallization space by introducing various lipid A/LPS moieties. Initial success and major crystallization leads were achieved using Re LPS. Crystals of MsbA (ATP–Vi) with Re LPS were grown in conditions as described above (shown in Fig. 1*b*). After screening and collecting synchrotron data from several crystals, we determined that the diffraction power of the crystal was limited to an average resolution of 6 Å with intensities observed to ∼5.5 Å (highest). The average unit-cell parameters were a = 260, b = 117, c = 170 Å, β = 121° and the space group was $C2$. Again, various methods were employed to improve crystal diffraction with no significant results.

With the improvement in order upon complex formation of MsbA and Re LPS, we pursued this lead and sampled the wide variation in size of the oligosaccharide domain of LPS. Crystals of MsbA (ATP–Vi) with Ra LPS were grown in a similar manner as those with Re LPS and appeared with approximately the same regularity (Fig. 1*c*), although the diffraction was significantly better for the Ra LPS-containing crystals, with data sets collected to ∼4.2 Å and spots observed to 3.6 Å. The difference between Re and Ra LPS is shown schematically in Fig. 1(*d*), highlighting the significant size difference in the oligosaccharide domain. Over ten data sets of diffraction data were collected for MsbA (ATP–Vi)–Ra LPS crystals in order to ensure the highest possible quality of diffraction data for crystallographic studies. The average unit-cell parameters were a = 271, b = 122, c = 178 Å, β = 121° and the space group was $C2$.

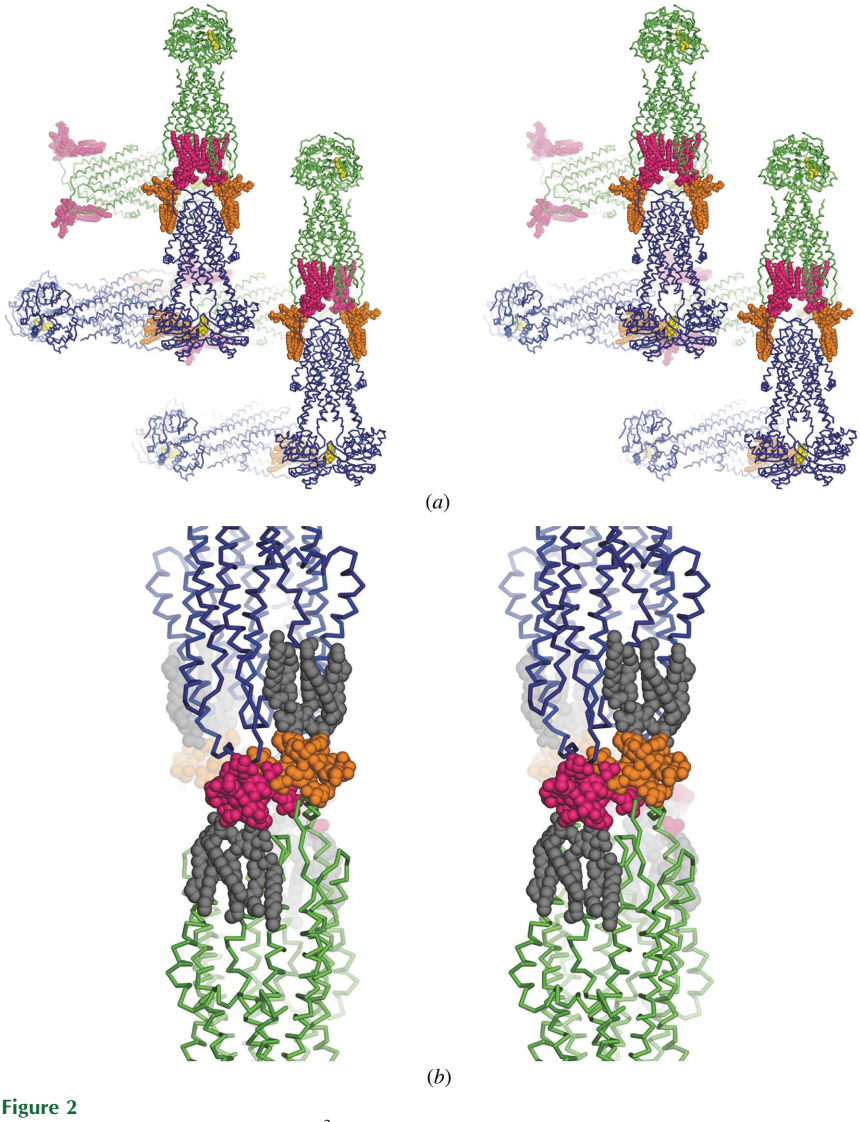

Figure 2
Crystal packing of MsbA (ATP–Vi, Mg^{2+})–Ra LPS. (*a*) The crystal packing and LPS interaction shown in stereo. The head-to-head interaction of the two crystallographic symmetry-related copies of MsbA (green and blue) is mediated by the LPS (purple and orange). ADP is shown in yellow. (*b*) The interdigitated LPS oligosaccharide domains mediate the crystal packing. Oligosaccharide domains are colored purple and orange; the fatty-acid acyl chains are colored grey.

3.2. Ra LPS provides the 'glue' in the crystal packing

The Ra LPS molecules bind to the extracellular side of the transmembrane domain of the MsbA dimer, with the sugar groups from one dimer interacting with the sugar groups from a crystallographic symmetry-related dimer (Fig. 2*a*). The LPS oligosaccharide domains interact extensively and mediate the entire head-to-head interaction of the symmetry-related MsbA dimers. Interestingly, the Ra LPS-specific sugar groups form most of the contacts at this lattice juncture (Fig. 2*b*). The similar unit-cell parameters and identical space group for the Re and Ra LPS crystal forms strongly indicate similar crystal packing. However, the absence of these additional sugar groups that are present in Ra but not in Re LPS is likely to be the reason that the Re LPS complex crystals have a smaller unit cell and are not as well ordered as the Ra LPS complex crystals. In the absence of the additional sugar groups, the Re LPS interactions are not as sterically or chemically stable and therefore the crystal packing is less ordered. The interacting polypeptide segments that facilitate the crystal lattice contacts are the two extracellular 1 (EC1) loops from dimers related by the crystallographic twofold. We speculate that the interactions of these loops alone would be much less stable owing to the inherent flexibility of these loops as observed in previous structures.

4. Conclusion

The use of a natural lipid substrate, in this case lipopolysaccharide, in the crystallization of MsbA was critical to the overall stability of the crystals and led to increased diffraction resolution. One of the most important findings is that

variation of the size of the oligosaccharide domain of the LPS has a direct effect on the diffraction resolution because of its extensive role in the crystallographic packing interface. We conclude that for the LPS to play such a dramatic and direct role in the crystal packing, it must bind to the protein before crystal lattice formation. Interestingly, the improvement in diffraction limits was not observed with the addition of non-substrate lipids. This would imply that although the addition of lipid to membrane-protein crystallization mixtures may have some positive effects in certain cases, it may be more important to focus on specific lipids in others. In the case of membrane proteins with specific lipid-dependent function, such as MsbA, identification of the lipids to use in crystallization is relatively straightforward. However, tightly associated lipid molecules that play a role in protein stability are also important and more difficult to identify and this is the current frontier in lipidomics (Han & Gross, 2005). These lipids may be separated from the protein in the latter stages of purification and added back during the crystallization stage. Assuming that the lack of lipid does not cause denaturation, the additional lipid can have a positive effect on both stability and crystallization. An analysis of lipid binding to a target membrane protein as well as the identification of endogenous lipids copurified at an early stage of purification should prove useful. In summary, the use of specific lipid substrates in membrane-protein crystallography represents a significant variable to help improve the quality of crystals.

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