Three-dimensional Structure of Wza, the Protein Required for Translocation of Group 1 Capsular Polysaccharide across the Outer Membrane of *Escherichia coli* *

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**Konstantinos Beis**, Richard F. Collins§, Robert C. Ford§, Alhaji B. Kamissî, Chris Whitfield¶, and James H. Naismith**

*From the Centre for Biomolecular Sciences, University of St. Andrews, North Haugh, St. Andrews, Fife KY16 9ST, United Kingdom, §Department of Biomolecular Sciences, University of Manchester Institute of Science and Technology, Manchester M60 1QD, United Kingdom, and ¶Department of Microbiology, University of Guelph, Guelph, Ontario N1G 2W1, Canada

Wza is a highly conserved multimeric outer membrane protein complex required for the surface expression of the serotype K30 group 1 capsular polysaccharide in *Escherichia coli*. Here we present the first three-dimensional structure of this type of polysaccharide exporter at a 15.5-Å resolution obtained using single particle averaging on a dataset of cryo-negatively stained protein. Previous structural studies on purified Wza have revealed a homo-oligomeric ring structure that is most probably composed of eight subunits. Symmetry analysis of the three-dimensional structure combined with biochemical two- and three-dimensional crystallographic data strongly suggest that Wza is an octameric complex with a C4 quasi-rotational symmetry and is organized as a tetramer of dimeric subunits. Wza is best described as a stack of two 4-Å high rings with differing diameters providing a mushroom-like aspect from the side. The larger ring has a distinctive square shape with a diameter of 115 Å, whereas the smaller is almost circular with a diameter of 90 Å. In the center of the complex and enclosed by the four symmetrical arms is a small elliptical cagelike cavity of ~40 Å in diameter. The central cavity is effectively sealed at the top and bottom of the complex but has small inter-arm holes when viewed from the side. We discuss the structure of this complex and implications in the surface translocation of cell-surface polysaccharide.

*Escherichia coli* produces >80 structurally and immunologically distinct capsular polysaccharides termed K antigens (1). These polymers vary in composition, linkage specificity, and substitution. Capsules are important virulence determinants that enable pathogenic bacteria to evade or counteract the unspecific host defense during the early (preimmune) phase of infection. They also interfere with the action of complement and phagocytes, although this effect is generally transient and overcome by capsule-specific antibodies in the immune phase of the host defense. In some cases, capsules are not immunogenic (or are poorly immunogenic) as a result of a structural mimicry or identity with host material, and these capsule types are correlated with highly virulent isolates. Examples include the group 2 of K1 and K5 serotypes (2). The capsular K antigens of *E. coli* are classified into four categories (denoted as groups 1–4) based on their surface organization, their assembly mechanism, the organization of their biosynthetic gene loci, and the regulation of their expression (3). However, in terms of their assembly, there are only two pathways used in *E. coli*, and the capsules of groups 1 and 2 provide the prototypes. Essentially identical pathways are found in other bacterial species (for review see Ref. 3). In all of the cases, capsular polysaccharides are synthesized at the inner membrane and must be translocated across the outer membrane for final assembly on the cell surface.

Group 1 capsules have a relatively low charge density and contain hexuronic acids as acidic components (3). Group 1 K antigens are found in two forms on the cell surface. Short K-antigenic oligosaccharides are attached to lipid A in a form resembling lipopolysaccharides (5). In contrast, high molecular weight polymers forming the capsular structure are not attached to lipid A and are assembled on the cell surface in a translocation pathway independent from lipopolysaccharides (6). Group 2 capsules have a high charge density and may contain hexuronic acids, N-acetylenuraminic acid, or 2-keto-2-deoxymanno-octulosonic acid as acidic components (2, 7). Group 2 polysaccharides have phosphatidic acid at their reducing termini, and this is thought to act as the surface anchor (8, 9).

The oligosaccharide repeat units of the group 1 K30 antigen from *E. coli* (O9a:K30) are assembled on an undecaprenylphosphate carrier, and the glycosyltransferases have been identified (6). They are then transferred across the inner membrane by an unknown process involving Wzx and polymerized at the periplasmic face of the membrane by the polymerase Wzy. These proteins are the characteristic components of a Wzy-dependent assembly pathway and are best studied in the assembly of lipopolysaccharide O antigens (reviewed in Ref. 10). High level polymerization of group 1 capsular polysaccharide requires phosphorylation of an inner membrane tyrosine autokinase, Wzc (11). Wzc is dephosphorylated by its cognate phosphatase, Wzb, and this protein is also essential for capsule assembly (11, 12). Two outer membrane proteins are involved in K30 assembly. The Wzi β-barrel protein acts late in the assembly process and appears to be involved in surface association (but not synthesis) of capsular polymer, although its exact role is unknown (13). Wza proteins are lipoproteins that form multimeric outer membrane complexes that are involved in translocation of the capsular polymer across the outer mem-

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**A Biotechnology & Biological Science Research Council (UK) Career Development Fellow. To whom correspondence should be addressed. Tel.: 44-1334-463792; Fax: 44-1334-467229; E-mail: naismith@st-andrews.ac.uk.**

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brane (6, 13). Group 2 capsules are synthesized by a hetero-
oligomeric membrane-bound biosynthetic complex on the inner
face of the cytoplasmic membrane by the sequential action of
glycosyltransferases that elongate the polysaccharide at its
non-reducing end (14). Translocation across the inner mem-
brane characteristically requires an ABC-2 (ATP-binding cas-
sette) transporter (3, 15, 16). Although some group 2 genetic
loci encode homologs of Wza (6) and models for export have
been proposed (16), little is known regarding their structure
function. The outer membrane component involved in the
translocation of group 2 capsular polysaccharides in E. coli is
unknown.

The Wza protein assembles into a stable oligomeric complex,
which can form two- (13) and three-dimensional crystals (17).
Negatively stained two-dimensional crystals revealed ringlike
multimers with an average outer diameter of ~9–10 nm and
central stain-excluding region of ~2–3 nm (13). Single particle
analysis of the same detergent-solubilized material identified a
putative C8 rotational symmetry, suggesting that the Wza-His₆
complex is octameric (13). At a gross structural level, Wza
multimers resemble the secretins such as Pilq (18), PilV (19),
PuIΔ (20), XcpQ (21), and YscC (22), which export proteins in
Neisseria sp., Pseudomonas aeruginosa, Klebsiella oxytoca, P.
aeruginosa, and Yersinia sp., respectively. However, these sim-
ilarities do not extend to the primary sequence features (13).
Nevertheless, the higher order structural similarities suggest
that detailed studies of one protein will provide information on
the biology of the other systems.

Electron microscopy (EM)¹ has a particular value for exam-
ining large-scale conformation changes within oligomeric pro-
tein complexes (23). We aim to use EM to investigate how the
components interact during polysaccharide export. As the nec-
essary first step, we have determined the three-dimensional
structure of native Wza multimers using cryo-negative staining
combined with single particle analysis (22, 23) to 15.5 Å. This
technique imparts a higher contrast (for a relatively small
complex) than is possible for particles observed by cryo-EM yet
retains the advantages of sample preservation (24). Our anal-
ysis is the most detailed structural information currently avail-
able for this class of exporter protein.

MATERIALS AND METHODS

Expression and Purification of Wza—The expression and purification
procedures that were used to isolate recombinant Wza have been de-
scribed elsewhere (25).

Cryo-negative Staining—Carbon-coated copper grids (number 400)
were inverted on the surface of a 10-µl droplet of Wza (5–10 µg/ml in 25
mM Tris, pH 7.5, 80 mM NaCl, 0.008% (w/v) dodecyl-β-maltopyranoside
(DDM)) for several minutes and then blotted sequentially for 5 s on a
Whitman filter paper until dry. Grids then were placed on a 20-µl
droplet of freshly prepared 5 or 10% (w/v) ammonium molybdate, 1%
(w/v) trehalose for several seconds, blotted briefly, and immediately
frozen to liquid nitrogen temperatures in an Oxford system cryo-stage.
Table I summarizes all of the additional information relevant to trans-
mission electron microscopy low dose cryo-data collection and image
processing.

Image Processing and Structure Calculation—The three-dimensional
structure of Wza was calculated using common line projection matching
methods implemented in EMAN. The initial dataset of 2735 Wza par-
ticles was selected interactively using either the graphic interfaces of
SPIDER (WEB) (26) or EMAN (BOXER) (27). The contrast transfer
function for each particle in the dataset was determined using the program
CRISP (28) and corrections for anisotropies were applied using SPIDER.
Data then were converted in a format suitable for
EMAN (27), and the contrast was normalized. The selected particles
were low-pass-filtered initially to 10 Å and centered in their boxes
relative to a reference-free global average. A set of reference-free class
averages then was generated corresponding to Wza positioned in mul-
tiple particle orientations. By following established procedures in the
EMAN software suite, a preliminary three-dimensional model was de-
termined from class averages that represented distinct views of the Wza
complex with a C4 symmetry applied. The relative orientations of the
characteristic views were determined using a Fourier common-line
routine, and the resulting averages were combined to generate the
preliminary three-dimensional model. The three-dimensional structure
was refined subsequently using eight rounds of iterative projection
matching with each refinement assessed by examining the convergence
by comparison of the Fourier shell correlation (FSC) of the three-
dimensional models generated from each iteration. The final three-
dimensional volume was converged fully after eight rounds of iterative
refinement.

Symmetry Analysis of Wza—Rotational symmetry in volumes was assess-
ed initially in EMAN using STARTCSYM. Unsymmetrized parti-
cle averages (with the strongest specified rotational symmetry) were
compared with a symmetrized version for consistent projection features
and improved SNR. For C4 symmetry, both projections were similar.
The assignment of C4 symmetry then was validated independently in
SPIDER (23) using a variation on rotational power methods and self-
orientation analysis (15).

Resolution Determination and Calculation of Variance Maps—Resolu-
tion was determined by FSC analysis by comparing the correspond-
ence in reciprocal space of two subaverages generated from half of the
final dataset. Using the same subvolumes, a variance map for the final
three-dimensional structure was calculated to illustrate the regions of
the structure with a high degree of variance (30).

Two-dimensional Crystallization of Wza and Image Processing—
Two-dimensional crystals of Wza were grown using a hanging-droplet
method as described previously (31) and negatively stained with 4%
(w/v) uranyl acetate. Electron microscopy was carried out under low
dose conditions on a Tecnai 10 transmission electron microscope oper-
ating at 100 kV. Images were recorded on Kodak SO-163 film and
digitized on a UMAX Power Look 3000 densitometer at 9.7 Å/pixel at
the specimen level. Lattice unbending and contrast transfer function
corrections were applied as described earlier (31) using the Medical
Research Council UK-Laboratory of Molecular Biology software suite
(32, 35). Projection maps were generated using the CCP4 software (34).

RESULTS

Cryo-electron Microscopy of Wza—A typical field of view contain-
ing cryo-negatively stained Wza multimers within the cryo-
preservative staining layer showed complexes positioned in
multiple orientations (Fig. 1A). Compared with previous obser-
vations of negatively stained Wza single particles (35), a vari-
ety of views were readily apparent with a lower image contrast
compared with the uranyl acetate-stained specimens. The most
frequently observed structures were the distinctive high con-
trast rounded square-shaped projections with an obvious axis
of rotational symmetry and variously tilted variations of these
views. A rounded, tapering rectangular two-handed projection
also was encountered frequently. Two-dimensional projection

¹ The abbreviations used are: EM, electron microscopy; FSC, Fourier
shell correlation.
classification of the particles corresponded to different orientations of the Wza complex with respect to the electron beam as shown in Fig. 1B. This sample of views also demonstrates a good coverage of the three-dimensional space required for projection matching.

Rotational Symmetry of the Wza Oligomer—A tetrameric rotational symmetry assignment (C4) was used for calculating subsequent volumes of Wza based on several rationales. First, the observations of the data recorded here in cryo show that the particle top view (positioned above the axis of rotational symmetry) has a distinctive square appearance and suggest that the basic symmetrical element of Wza is tetrameric. Second, previous EM data have suggested that Wza has an octameric organization; however, the calculation of a three-dimensional structure using C8 symmetry (rather than C4 symmetry) produced class averages that were visually dissimilar to the raw data and produced a lower resolution three-dimensional structure, which would not converge properly (data not shown). Equivalent comparisons with other symmetries were only reasonable for C1, C2, and C4 datasets. Fig. 1B shows some of the 85 Wza two-dimensional projection classes and the corresponding back projections from the final three-dimensional symmetry model (lower panel). Box size = 171 Å². C, self-rotational plot of the Wza oligomer calculated from a 3.0-Å resolution three-dimensional crystal dataset (17).

EM Structure of Wza

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Fig. 1. Cryo-negative-stained detergent solubilized Wza oligomers. A, a micrograph of Wza complexes in 1% (w/v) trehalose and 10% (w/v) ammonium molybdate recorded under cryo-conditions. The field displayed was recorded at a defocus of ~3.4 μm, and the contrast has been enhanced for presentational clarity. Projections of Wza multimers presenting multiple orientations are highlighted in square boxes. Scale bar = 500 Å. B, a montage of different orientation classes produced by single particle averaging of the Wza multimer dataset. Two-dimensional class averages are displayed (top panel) with corresponding back projections from the final three-dimensional symmetry model (lower panel). Box size = 171 Å². C, self-rotational plot of the Wza oligomer calculated from a 3.0-Å resolution three-dimensional crystal dataset (17).
tein mass of 276 kDa and is consistent with an oligomer containing eight Wza molecules. Finally, self-rotation peaks from three-dimensional crystal data (17) were calculated using the CCP4 program suite (37). The data showed both C4 and C8 rotational peaks as shown in Fig. 1C. These data are all entirely consistent with a complex possessing quasi-symmetry with a C4 rotational axis. In turn, these data strongly suggest that the octameric Wza is a tetramer composed of Wza dimer subunits.

Three-dimensional Structure of Wza—Six rounds of angular reﬁnement were applied to 2735 Wza particles to determine the final three-dimensional structure. Surface-rendered representations of the three-dimensional structure of Wza viewed from the bottom, side, and top are shown in Fig. 2A. The Wza complex can be divided into two layers. A larger (115 Å) layer is located at the top with a smaller (90 Å) layer at the bottom. Both layers are formed by four tightly associated subunits with no indication of a pore or channels between them at the top and bottom faces. The bottom layer is rotated ~20° relative to the top layer, giving an overall twist to each subunit in the tetramer. Although the top layer is relatively flat on its upper surface, the lower layer tapers into a rounded tip made up of the four subunits. The side views reveal that these two layers are joined by four bridging densities allowing the 20° rotational twist between the two layers. The complex has an overall height of ~85 Å in this side view. For scale, this is roughly twice the thickness of an average lipid bilayer. In between the connecting densities (or arms) are four approximately circular holes of ~35 Å in diameter that connect to a central cavity. This cavity is roughly elliptical in shape and is ~20 Å in height and 40-Å-wide at the widest points. The extent of the central cavity and its internal features within the complex also are shown in Fig. 3. At this resolution, we are unable to identify monomer units within the structure.

Resolution and Variance with the Three-dimensional Structure—Fig. 3A shows the resolution estimation by FSC for the final volume. Here, two separate three-dimensional structures were created from either even- or odd-numbered particles. Hence, each structure represents only 50% of the full dataset. The extent of structural similarity then is compared in Fourier space by correlation analysis (38), and the data are averaged over Fourier shells in three-dimensional reciprocal space to allow a plot of correlation coefficient versus global resolution. Generally, the accepted methods for resolution-limit estimation have recommended that a FSC threshold of 0.5 (38) be employed, although this may be a conservative assessment (39). An FSC threshold of 0.5 provides a resolution estimate of 15.5 Å for the Wza structure consistent with the three-dimensional variance map analysis. As shown in Fig. 3B, some small regions of high variance (rendered at 3σ above the mean variance) are well outside the periphery of the molecule. This is probably because of a lack of consistency in staining. At the bottom of the “arm” densities connecting the top and bottom layers, there is evidence of high variance within the protein density and this may reflect flexibility of the subunits in this region. The three regions of high variance along the 4-fold axis of rotational symmetry (dashed line) are intriguing and are potentially indicative of conformational flexibility that would be necessary to open a polymer translocation pore in the membrane. In this respect, the top and bottom layers behave differently with the top layer showing a high variance at the center of the layer, whereas the bottom layer displays a low variance in the center.

Two-dimensional Crystals of Wza—The structural analysis and interpretation presented above were verified independently using an electron crystallographic approach. Two-dimensional crystals of the detergent-solubilized Wza complex were obtained using a hanging-droplet method (31), and Fig. 4A shows an image of typical crystals after negative staining. The crystals are composed mostly of single layers of Wza molecules, but occasional patches with two or more overlapping layers can be observed (arrows in Fig. 4A). In addition, small defects in the crystal lattice were observed (boxed/zoomed area) where Wza particles in different orientations were incorporated into the crystal. Fig. 4B shows a computed Fourier transform of a well ordered area of the Wza crystal subjected to three rounds of lattice unbending. The transform shows systematic absences along the (h,0) axis, indicative of a screw axis along the crystallographic a direction. The calculated projection map (Fig. 3C) (with no crystallographic symmetry applied) shows two Wza tetramers in the unit cell offering further support for the presence of a screw axis. It seems probable that the crystals form in the P121, plane group with the two Wza tetramers in the unit cell alternately being oriented facing upward and downward. Because there is some differential staining of the two sides of the crystal, there is a significant deviation from a perfect P121, relationship. This provides one explanation as to why one of the Wza tetramers appears to be slightly smaller in projection. Presumably, this molecule has its smaller (90 Å) layer more contrasted by stain, and hence, this contributes more to its projection. The overall size and shape of each molecule in the unit cell is consistent with the single particle averaging described above. Each molecule is ~120 Å with a central stain pocket of 30–40 Å in diameter. There is no indication from this crystallographic study of an 8-fold symmetry signal, in agreement with the application of C4 symmetry for the single particle averaging procedures. Fig. 4, panels D and E, shows the two molecules in the unit cell after the application of non-crystallographic 4-fold symmetry.

DISCUSSION

Three-dimensional Structure of Oligomeric Wza—The mushroom-like Wza-multimeric complex is divided into two discrete layers and appears to be formed by eight monomers with 4-fold rotational symmetry, suggesting a tetramer of dimers. A sim-
ilar architecture and C4 symmetry have been observed in other membrane proteins, e.g. the inositol 1,4,5-triphosphate receptor (40) and the Shaker channel (41), although in the Shaker channel, the lower ring shows a smoother surface relief and has a pronounced funnel shape. In these examples, the structural data show that the larger ring is observed in the membrane-embedded portion of these structures. By analogy, we would suggest that the larger ring of the Wza complex is embedded in the membrane. The detailed structure provided by this study also allows some structural comparison between Wza and the protein export "superfamily" secretins and highlights some important differences. PilQ from Neisseria meningitidis forms a large (∼1 MDa) oligomeric outer membrane complex that is responsible for the extrusion and subsequent retraction of adhesive-type IV pili. Similar to Wza, the PilQ secretin also is C4-symmetric and both PilQ and Wza complexes have four arms that project from the bottom layer, twisting around a central cavity and meeting to form a closed dome-shaped “cap” on the top of the complex. However, the overall dimensions and internal cavity of PilQ are considerably larger than Wza, and the complex is probably formed from 12 monomers rather than eight. When viewed from the side, PilQ also has a three-layered structure. The internal cavity of PilQ is also almost completely sealed on all of the sides, whereas the cavity of Wza has access channels. The pIV multimer, which secretes filamentous bacteriophage, has 14 monomers in a distinct three-band structure similar to PilQ. However, the central cavity of pIV (60–88 Å in diameter) is cut in two by the central (M) ring of the complex (42). In contrast to Wza multimers, the pIV-oligomeric structure shows a channel opening at each end, perhaps connecting

Fig. 3. FSC resolution estimate of the final Wza complex three-dimensional volume and structural variance within the volume. A, the FSC data were calculated on the final iteration of the structure at 1σ above the mean density with a C4 symmetry applied. B, variance map (shown in pink at 3σ above the mean variance) superimposed on a central 44-Å-thick slab of the Wza-multimer structure (gray surface) viewed from the side. High variation within the three-dimensional structure occurs in the arm regions and also along the 4-fold axis of rotational symmetry (dashed line). For clarity and to allow a direct comparison with the map for the Wza multimer, variance maps were first low pass-filtered to 15 Å. Scale bar = 50 Å.

Fig. 4. Two-dimensional electron crystallography of Wza. A, two-dimensional crystal of Wza multimers negatively stained with 4% (w/v) uranyl acetate. Arrows indicate the areas where protein has started to crystallize in more than one sheet. The dotted box magnifies ×2 an example area of mosaic nucleation where the side and partial side views have been incorporated into the two-dimensional lattice. Scale bar = 500 Å. B, computed Fourier transform of an image of a crystalline area. The principal crystallographic axes (h,k) in reciprocal space are indicated, and the size of the boxes are an indication of the signal/noise of the reflections (33). The first circle is at a resolution of ∼32 Å. Reflections to ∼25 Å can be observed in the transform. C, two-dimensional projection map of the crystal of Wza multimers using contours to delineate protein density (contours begin at 0.5σ above the mean density and are separated in steps corresponding to 0.2σ). No crystallographic symmetry has been applied. The crystallographic axes are shown: a = 158 einsteins; b = 264 Å; γ = 90°. D and E, projection maps of the two molecules in the unit cell after non-crystallographic C4 symmetry has been applied. The top complex is slightly smaller in projection, probably because of differential staining (see “main text”). Scale bars = 50 Å.
the periplasm and external milieu. The modeling of the PuD secretin revealed a C12-symmetrical cylindrical structure with a continuous and open channel visible through the entire length of the protein complex. Although clearly visible under the conditions of the EM experiment, conductance measurements indicate that the pore is gated (43). However, transmission electron microscopy analysis of proteolytically digested PuD suggests that the three-dimensional structure may be more similar to that of pIV (20).

Oligomeric Arrangement of Wza—Quasi-symmetry is well characterized for viral capsid proteins but has been observed relatively infrequently for other oligomeric assemblies. Notably, quasi-symmetry is a feature of membrane and membrane-associated protein complexes such as PilQ. The PspA AAA+ adaptor protein assembly forms a C9-symmetric ring associated with the inner membrane (44). The molecular mass of the complex is ~1 MDa and suggests that each of the nine rotational units is a tetramer. The structural significance of these quasi-symmetrical arrangements is unclear, but the symmetry mismatch has a functional significance because the export substrates typically will have either no symmetry or a different symmetry compared with the exporter itself.

Implications for K Antigen Synthesis—In the working model for group 1 capsule translocation, the large ring of the Wza multimer is membrane-embedded. Because its depth almost exactly matches the thickness of the membrane, it would be occluded completely. Consequently, our prediction is that the entire smaller ring is exposed in the periplasm. An alternative positioning of the complex in the membrane would require the side openings to be inside the lipid bilayer, although this seems unlikely. The structure is entirely consistent with a model in which Wza interacts with K antigen synthesis components located at the inner membrane. Preliminary biochemical evidence suggests protein-protein interaction with the inner membrane protein Wzb (13). This would be similar to the interactions of secretins (such as MxiD) with the inner membrane components in type III needle complex structures (45, 46). There is an obvious analogy to the ArcAβ-ToCl drug efflux pump from *E. coli* (47). The ToCl can be coupled to either an ABC transporter or an antiporter depending on the export substrate (48, 49). The Wza system does not require an ABC transporter for translocation of the K antigen.

The Wza multimeric structure suggests the complex is closely to the external environment, but access to the complex cavity may be achieved via the “side” holes from the periplasm. It may be that this is the entry point for the capsular polysaccharide with the export point existing through the middle of one of the two rings. We have proposed already that it is the large ring that is inserted in the membrane, making this the exit point in the hypothetical model. Under the conditions used for preparation and electron microscopy, the structures show no open channel through this ring. However, because both Wza and secretins act as substrate-specific portals across the outer membrane, it appears that we are observing the closed form of Wza multimers. In order for the substrate to thread through Wza, the membrane-embedded layer would have to open up. Given that the Wza substrate is large (100 kDa), this would require significant conformational change in the upper ring. As discussed above, the experimental data suggested that there was indeed some flexibility in the larger (but not smaller) ring, consistent with our hypothesis for opening and closing of the larger ring. This gating may be triggered either by substrate or interaction with another protein. One candidate is Wzc, a protein that can be cross-linked to Wza in a higher order complex.