STRUCTURE OF THE HEMOLYSIN E (HlyE, ClyA, SheA) CHANNEL IN ITS MEMBRANE-BOUND FORM*
Svetomir B. Tzokov, Neil R. Wyborn, Timothy J. Stillman, Stuart Jamieson1, Nadine Czudnochowski2, Peter J. Artymiuk, Jeffrey Green, Per A. Bullough+
From the Krebs Institute for Biomolecular Research, Department of Molecular Biology and Biotechnology, The University of Sheffield, Sheffield, UK
Running Title: Structure of the Hemolysin E Channel
+Address correspondence to: Per A. Bullough, Department of Molecular Biology and Biotechnology, The University of Sheffield, Firth Court, Western Bank, Sheffield S10 2TN, UK; Tel: +44 114 22 24245; Fax: +44 114 22 2800; Email: p.bullough@sheffield.ac.uk

Hemolysin E (HlyE, Hemolysin E, Cytolysin A, ClyA, SheA) is a pore-forming protein toxin isolated from E. coli. The three-dimensional structure of its water-soluble form is known, but that of the membrane-bound HlyE complex is not. We have used electron microscopy and image processing to show that the pores are predominantly octameric. Three-dimensional reconstructions of HlyE pores assembled in lipid/detergent micelles suggest a degree of conformational variability in the octameric complexes. The reconstructed pores are significantly longer than the maximum dimension of the water-soluble molecule indicating that conformational changes occur on pore formation.

Hemolysin E (HlyE3) is a pore-forming toxin (1-3), not related to any of the other known pore-forming hemolysins. Expression of HlyE in the absence of the well-characterised RTX toxins is sufficient to give a haemolytic phenotype in E. coli (4) and it is known that HlyE is present in pathogenic strains of E. coli, including E. coli 0157 (5). The atomic resolution structure of the 34 kDa HlyE monomer in its water-soluble form, (Fig. 1A) reveals a bundle of four major α-helices, with a small surface-exposed hydrophobic β-hairpin (6) at the “head” end of the structure, and the N- and C-termini at the “tail” end. In the presence of a phospholipid membrane HlyE forms moderately cation-selective pores predicted to have a diameter of ~30 Å, as measured by lipid bilayer conductance experiments and osmotic protection assays (1,7,8). Direct visualisation of pores by electron microscopy (EM) of negatively stained membrane-reconstituted HlyE, suggested outer diameter of 70–90 Å and inner diameter of 40–50 Å. There was also evidence of some variability in pore size in the form of a minor population of larger pores (6). Given the absence of detailed structural data on the pore complex, a conservative model of the pore was proposed on the basis of the available X-ray and EM data (6).

In an attempt to structurally characterise the HlyE pore, we have performed EM on isolated pore assemblies combined with single particle image averaging. We present a three-dimensional (3D) EM structure of the HlyE pore in mixed lipid/detergent micelles. The resolution is sufficient to reveal individual subunits and allows for the first time a comparison of subunit structures in aqueous and membrane-bound forms of the HlyE protein.

EXPERIMENTAL PROCEDURES

Protein Isolation and Characterisation – HlyE was purified as previously described (6,9). Briefly, clarified French press cell extracts were applied to a GSH-Sepharose 4B column (Amersham Biosciences) and HlyE released by thrombin treatment. Truncated HlyE was produced by prolonged (≥45 weeks) incubation of wild type HlyE in thrombin cleavage buffer (TCB, 25 mM Tris HCl at pH 7.5, 2.5 mM CaCl2, 100 mM NaCl) at 4°C, and used without further treatment. All protein solutions were stored at -20°C after their isolation or, in the case of the truncated pores, their preparation.

Samples were characterised by SDS-PAGE and N-terminal amino acid sequencing, as described previously (6,9), as well as by BN-PAGE (10). MALDI-TOF analyses of the truncated HlyE were carried out at Micromass and Applied Biosystems (Warrington).
Electron Microscopy of HlyE Pores in n-Octyl-β-D-Glucopyranoside Micelles – Five µl of a 1 mg/ml solution of purified HlyE in TCB (25 mM Tris HCl at pH 7.5, 2.5 mM CaCl₂, 100 mM NaCl) were added to 2 µl 6% w/v n-octyl-β-D-glucopyranoside (βOG) (Calbiochem) in TCB diluted with 3 µl TCB, to a final protein concentration of 0.5 mg/ml and detergent concentration of 1.2% (2x critical micelle concentration, CMC). After a brief sonication at room temperature, the solution was incubated at 37°C for 30 min and then either immediately used for EM, or stored for up to 4-5 weeks at 4°C.

Samples for data collection were adsorbed on glow discharged carbon-coated copper grids and stained with 0.75% (w/v) uranyl formate. Electron micrographs were recorded at a nominal magnification of 39,000X and underfocus of 4000-9000 Å on a Philips CM 100 electron microscope, using a Gatan MultiScan 794 CCD camera (Fig. 2A). The magnification was calibrated by imaging of negatively stained catalase crystals (Agar).

Determination of the Pore Symmetry – All alignments and multivariate statistical analyses (MSA) were carried out using the IMAGiC-5 software package (Image Science Software GmbH) (11,12). After direct alignment to the total average and cycles of multi-reference alignment (MRA) to selected averages, approximately 650 images of top views were selected from an initial set of over 1400 images of top and side views of HlyE pores in βOG. "Top views" were images of pores viewed down the putative axis perpendicular to the membrane plane, while "side views" were images of pores viewed parallel to the putative membrane plane. For determination of the averages, used in the MRA and for selection of the images, three different criteria were used: high 'overall quality' as defined in IMAGiC, high total number of images in the corresponding MSA class, and representation of intact circular complexes.

The selected set of top views was subjected to additional cycles of MRA with application of symmetries ranging from 6- to 12-fold to all references. This produced three class averages of different sizes, with no symmetry enforced: "big", consisting of 84 views (13%), "medium", consisting of 375 views (58%) and "small", consisting of 72 views (11%). Finally, images within each of these size groups were aligned by cycles of direct alignment to the total average of the group (with no symmetry enforced) (insets, Fig. 2B-D). After the final alignment, rotational power spectra (13) of the total average were determined in each subset (Fig. 2B-D) using the MRC package of programmes (14,15).

Electron Microscopy of HlyE Pores in Mixed Brain Lipid/β-OG Micelles – To determine the optimal conditions for imaging of individual pores in side views, protein, detergent and lipid were mixed in varying final concentrations, in the range of 125 µg/ml – 325 µg/ml for the HlyE, 0.3 – 3.8% for the β-OG (0.5x – 6x CMC) and 0.1 – 4 µg/ml for the brain total lipid extract (BTLE, Avanti Polar Lipids, Inc.) The mixtures were incubated at 37°C for 15 min and kept overnight at 4°C, before being examined. (Fig. 3). For example, 20 µl of 250 µg/ml HlyE in 1.2% β-OG (2x CMC), containing 4 µg/ml BTLE was prepared by mixing of 10 µl 500 µg/ml solution of HlyE in TCB with 10 µl 8.00 µg/ml BTLE solubilised with 2.4% β-OG in TCB. Stock solution of 4 mg/ml lipid solubilised in 2% β-OG and TCB was obtained by drying of 8 µl 20 mg/ml chloroform solution of BTLE in a stream of air at room temperature, followed by addition of 2.67 µl 30% water solution of β-OG, 29.2 µl deionised H₂O and 8 µl 5x TCB. The mixture was briefly vortexed after the addition of each component and finally sonicated for 10 min at room temperature. Micrographs were recorded under similar conditions as those used for protein in detergent-only micelles, but in low-dose mode.

Electron Microscopy of Truncated HlyE Pores – Truncated HlyE was loaded on grids either after dilution of the initial stock solution in TCB or after reconstitution experiments identical to those described previously for the wild type protein (6). Micrographs were recorded as above (Fig. 4A). The top view average (Fig. 4B) of the pores was obtained after cycles of direct alignment of 102 individual images to their total average, followed by rotational averaging of the total sum of aligned images. The side view average was obtained after cycles of direct alignment of 161 individual side view images. Mirror symmetry was enforced along the vertical; this is equivalent to cylindrical averaging (Fig. 4C).
Correlation analysis of the non-symmetrised total average of truncated pore side views was done by cross-correlating the average with side projections of rotationally averaged reconstructions of the full-length pore. The highest correlation was obtained for the non-association end of the full length complex, with a maximum value found for the "wide" pore reprojection. Correlation to "squat" pore reprojection was 98% and to "narrow" pore reprojection was 95% of the maximum. The highest correlation to the association end half of the reprojeciton was again maximum. The highest correlation to the "square" pore reprojection was 98% of the value found for the "wide" pore reprojection. The correlation to the "square" pore reprojection was 98% of the highest correlation obtained for the non-association reconstructions of the full-length pore. The highest correlation to the association end half of the reprojection was again maximum.

Three-Dimensional Reconstruction of Full Length Pores – A small set of ca. 100 side view images of HlyE pores in β-OG was subjected to direct alignment to a total average, six cycles of MRA to selected averages (selected by the criteria specified above) and two cycles of 3D reconstruction, reprojection and MRA, to give a preliminary 3D model of the HlyE pore. The latter was reprojected to produce a single reference for the direct alignment of a set of over 1000 individual images of side views of HlyE pores in BTLE/β-OG micelles. After MSA of the aligned images, MRA and a second round of MSA, selected averages were used for calculation of the initial low-resolution 3D reconstruction of the HlyE pore (Fig. 5). The initial reconstruction was refined through cycles of reprojection, MRA, MSA and 3D reconstruction. Projections were then sorted according to their shape and width into 4 groups and four 3D volumes were reconstructed, from the projections in each group as described above. The reconstructions were refined by further cycles of reprojection, MRA and MSA, until convergence was reached. Eight-fold symmetry was applied to all 3D reconstructions. From the initial set of 4 volumes, two merged during this further refinement leaving 3 unique reconstructions: "squat" pores, "wide" pores and "narrow" pores. To correct for the widening of the reconstructions due to the flattening of the negatively stained pores, the size of the top view reprojection for each volume was compared with the size of the averages produced during the analysis of the experimentally obtained top views (insets, Fig. 2B-D, Fig. 6B) by magnification alignment. A zoom factor of 0.9 was found for all reprojections and applied to compensate for the effects of lateral flattening.

Determination of the threshold density for surface rendering was carried out by examination of two-dimensional contour maps of cross-sections of the volumes. The threshold was determined at the midpoint of the maximum density gradient.

Current data do not allow determination of the absolute handedness of the channel, but for representation purposes, an arbitrary hand was chosen (Fig. 6C, D). Comparison of the cross-correlation coefficient between projections used for the reconstruction of the "narrow" pores and reprojections of the original and mirrored volumes of the other two reconstructions was used to determine the relative handedness of the “squat” pores and the “wide” pores (Fig. 6C).

RESULTS AND DISCUSSION

Pores in Lipid Vesicles – We have previously described EM experiments, in which HlyE pores reconstituted in lipid vesicles formed large domelike structures consisting of numerous closely packed pores (6). Similar clusters have been observed in electron micrographs of E. coli overexpressing HlyE in vivo (16). Precise analysis of these images is hampered by the variable size of the pores, the irregular nature of their close packing, and the curvature of the dome-like assemblies. This curvature appears to arise from the tapering V-shape of the individual pore assemblies, which are narrower at the end that binds in the membrane and wider on the exterior (Fig. 1B). Very rare observations of isolated pores in side view confirm this tapering (Fig. 1C).

In order to obtain more information on the structure and stoichiometry of the pore we attempted to collect many more images of isolated pores for single particle work.

Preparation of Pores for Single Particle Work – HlyE forms pores in a variety of detergents, but we found that in 1.2% β-OG (2x CMC) the proportion of clearly identifiable individual pores was highest. In these preparations, all images of pores suitable for image analysis were of ‘top’ views i.e. views down the putative axis perpendicular to the membrane plane (Fig. 2A), suggesting a preferred surface of interaction of the complex with the carbon film. Pores in ‘side’ view (views parallel to the putative membrane plane)
were generally seen in multilayered clusters (Fig. 2A).

Symmetry of the HlyE Pore - Analysis of Top Views – Average images were selected as references for MRA on the basis of the number of images making up each average, low variance and a fully intact circular structure. MRA was performed on selected top views using references with six- to twelve-fold rotational symmetry applied. Four cycles of MRA resulted in the generation of three class averages of differing size and symmetry (Fig. 2B-D: "big" (13%), "medium" (58%) and "small" (11% of all analysed top views). Rotational power spectra (13) showed conclusively that the "big" and the "medium" averages both had 8-fold symmetry, while the "small" average had 6-fold symmetry (Fig. 2B-D).

The existence of small but significant populations of pores with sizes considerably different from the overall average is in good agreement with the previous observations of pores reconstituted in lipid bilayers (6).

In some of the top views of pores (Fig. 1B, C, Fig. 2A, Fig. 3B) there is an apparent central density feature although this is not present in all of the top views. This density is comparable with that of the background and most likely arises from a preferential accumulation of negative stain along the walls of the pores. Moreover there is no indication of any central protein density in side views of pores (Fig. 5C, D).

Although previous EM, biochemical and biophysical data had suggested approximately eight HlyE subunits (6,9,16), the present work constitutes the first direct observation of the oligomeric structure of HlyE.

Analysis of Side Views – Side views of isolated HlyE pores were extremely rare in the electron micrographs of samples prepared in β-OG alone. Instead, the majority of side views were of pores in small clusters (Fig. 2A), unsuitable for image analysis. Extensive searches for alternative conditions, including addition of lipid to the protein/detergent mixture, produced a wide variety of different types of pore aggregates (Fig. 3), but very few individual pores. Mixtures of β-OG with defined phospholipids such as 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) or with POPC and cholesterol were tested but we found that mixtures of brain lipids and β-OG gave the most rapid and extensive assembly of pore even under these most favourable conditions, most of the pores in side views were found in aggregates. As a control we examined electron micrographs of detergent/lipid mixtures without addition of protein but none of these showed pores or pore aggregates found in the presence of protein.

Only one end of any individual pore assembly appeared to be involved in inter-pore aggregation, i.e. it is evident that the inter-pore interactions are either all head-to-head or they are all tail-to-tail. Thus the long "chain-like" pore aggregates and the pore pairs and the pore triplets in side views, are all built from protein pores which appear to be specifically oriented with respect to one another in a symmetrical manner with an apparent inter-pore “association end" towards the centre of the aggregate (Fig. 3, Fig. 5A). This phenomenon allows the relative orientations of the pores in side views to be readily determined.

Low Resolution Three-Dimensional Reconstructions – Attempts to obtain a 3D map of the pore by using the random conical tilt approach (17) on the top view pores seen in β-OG were unsuccessful, probably because of the extensive flattening of the pores in the axial direction perpendicular to the carbon support. Similarly, attempts to produce a 3D reconstruction by combining top views with side views were unsuccessful, probably because of the differential flattening of the pores in axial and lateral directions (18,19). Therefore, tomographic reconstruction from side view images (19,20), with application of the 8-fold symmetry expected from the predominant symmetry of top views, was used for generation of a low-resolution 3D map of the pore assembly. A small initial set of ~100 images of pores in side views was selected from micrographs of HlyE in mixed lipid/β-OG micelles and used to generate a starting 3D model of the HlyE pore. This was used as an initial reference for direct alignment of a larger set of >1000 side view images of individual pores (Fig. 5B), formed under conditions corresponding to image 4 of Fig. 3B. Cycles of refinement by projection matching were applied until convergence was reached with a final set of 3 different reconstructions of the HlyE pore (Fig. 5B-D), which were designated “squat” (184 images), “wide” (326 images) and “narrow” (348 images).
images). The resolutions of the reconstructions were estimated by Fourier shell correlation as ~30 Å (threshold value 0.5) (Fig. 5E).

Analysis of the Three-Dimensional Maps – The previous conservative model of the pore (6) suggested a tapered complex with external diameter of ~110 Å at the top and height of ~100 Å. Molecular envelopes of the three distinct species of pore identified also reveal slightly tapered assemblies. However, all 3D reconstructions are significantly larger than those of the existing model (Fig. 6A).

Flattening induced widening of the side view projections of cylindrical complexes is a known artefact in negatively stained specimens (18,19). When reprojections of the 3D reconstructions are compared with the top view averages of HlyE pores (Fig. 6B), it is evident that the top view reprojections of both the "squat" and the "wide" pores have a similar appearance to the "big" top view average – a ring of relatively well contrasted subunits. The subunits are less contrasty in the reprojection of the "narrow" pores, similar to the "medium" top view average. In all cases, however, the reprojections of the reconstructions from side view images are wider than the top view averages and have to be rescaled to be matched (Fig. 6F). Magnification alignment of the reprojections and the top view averages confirm that the "squat" and the "wide" pores best correlate with the "big" top view averages, with magnification correction factor of 0.9. The "narrow" pores correlate best with the "medium" top view average, also with correction factor of 0.9.

These results suggest that, because of the similarity of their diameters (Fig. 6A), top view reprojections of negatively stained "wide" and "squat" pores are indistinguishable (Fig.6B) and probably the group of the "big" top view averages, found during processing of top view images, is a mixture of individual views of "squat" and "wide" pores. Analysis of the reconstructions reveals also that the minimum internal diameter is located at the association end of the pore for all three reconstructions (Fig. 6A, C). The HlyE channel has been predicted to have a minimum diameter of ~30 Å (1,7,8). After correction for the effect of flattening, the minimum inner diameter of the "narrow" pores is found to be comparable, at ~35 Å.

Truncated HlyE – When incubated for several months at 4°C, HlyE pore preparations tend to partially hydrolyse, possibly due to traces of residual protease activity in the preparation. When observed by EM, the products of this partial hydrolysis are "short pores" (Fig. 4A), in which the top views (Fig. 4B) appear very similar to those of the complete pore, but the side views are significantly shorter (Fig. 4C) than the 120–140 Å long full length pores. Image analysis confirms that the side view average of truncated pores (Fig. 4C) has a diameter comparable to the wider end of the "squat" or "wide" pores (Fig. 5D). These data, coupled with the absence of the narrowing observed at the association end of the full pores (Fig. 4C), suggest that the truncated pores correspond to the non-association end of the full-length pores. This view is further supported by the absence of the pairwise associations characteristic of the full-length pores. Moreover, the images of the truncated pores show that they do not bind to any lipid bilayers tested (Fig. 4A), implying that it is the association end of the full-length pores that interacts with the membrane. Although only one band with mobility corresponding to molecular weight of ~8 kDa was apparent by SDS-PAGE of truncated HlyE, mass-spectrometry revealed that there were several hydrolysis products with molecular weights of approximately 8.07, 8.17, 8.28, 8.42, and 8.48 kDa. Two distinct N-terminal sequences were detected: SVLVDIKTL and SNTVKQANKD. The first is situated at the N-terminus of helix αF from which a peptide with molecular weight of ca. 8 kDa would include the whole of αF and the first half of αC (Fig. 4D). The second sequence commences in the middle of helix αF, and an 8 kDa peptide will include the second half of αF and the whole of αG (Fig. 4D). The absence of helix A in the truncated pore complex suggests that this part of the toxin structure, which would have an expected mass of ~4kDa, has also undergone proteolysis and is therefore not involved in or is peripheral to the hydrophilic end of the pore assembly. All these findings indicate that the complex in the truncated pores corresponds to the end of the full pore assembly that protrudes outside the membrane and also give further support for the view that it is the head domain, not present in these preparations, that is responsible for membrane interaction.
Single particle analysis of top views of the HlyE pore revealed the presence of at least two types of octameric assemblies and a small group of hexameric pores. Formation of active complexes with varying numbers of subunits is not unusual for pore-forming toxins: e.g. pneumolysin forms pores containing between 30 and 50 subunits (20). The observation of pores with different diameters, but having 8-fold symmetry is interesting and may indicate different forms of the HlyE channel corresponding to different functional conformations (e.g. pore and prepore complexes).

In the present study, we were able, for the first time, to observe individual side views of assembled HlyE channels (Fig. 5), allowing reconstruction of the HlyE pore. All three statistically different HlyE pore reconstructions have lengths significantly greater than the 100 Å of the water-soluble form of HlyE determined by X-ray crystallography. Even the shortest "squat" pores appear 20 Å longer than the X-ray structure, which is shown placed in the 3D map of the narrow pores in Fig. 6D, the orientation of the HlyE molecule being based on observations for the truncated pores and in agreement with previous indications that the head domain is embedded into the membrane. The observed elongated dimensions are consistent with our more recent EM studies of lipid-inserted complexes where we have rarely obtained side views of individual pores inserted into vesicles (Fig. 1C). These views show the hydrophilic domains extending ~80 Å beyond the surface of the bilayer. If the total thickness of the membrane is 50–60 Å (20-22), the expected full length of the HlyE channel would be as much as 130–140 Å, consistent with our measurements of the complexes in micelles. This suggests that the conversion of the water-soluble toxin to the membrane bound form may necessitate significant changes in conformation, as have been observed in other pore-forming toxins (23). For example, hinge movements within the "head" domain (Fig. 1A) could possibly elongate the molecule by ~25 Å, and indeed previous mutagenesis experiments suggest a possible movement between β1 and αD (9). Thus, the observations made here constitute important advances in our understanding of the mechanism of HlyE function, but it is clear that higher resolution data will be needed to fully characterise the major structural changes that are clearly involved in the transition between the water-soluble monomer and transmembrane pore complex.

REFERENCES

1. Ludwig, A., Tengel, C., Bauer, S., Bubert, A., Benz, R., Mollenkopf, H. J., and Goebel, W. (1995) Mol. Gen. Genet. 249, 474-486
2. Oscarsson, J., Mizunoe, Y., Uhlin, B. E., and Haydon, D. J. (1996) Mol. Microbiol. 20, 191-199
3. Green, J., and Baldwin, M. L. (1997) Microbiology 143, 3785-3793
4. Ralph, E. T., Guest, J. R., and Green, J. (1998) Proc. Natl. Acad. Sci. USA 95, 10449-10452
5. del Castillo, F. J., Leal, S. C., Moreno, F., and del Castillo, L. (1997) Mol. Microbiol. 25, 107-115
6. Wallace, A. J., Stillman, T. J., Atkins, A., Jamieson, S. J., Bullough, P. A., Green, J., and Artymiuk, P. J. (2000) Cell 100, 265-276
7. Ludwig, A., Bauer, S., Benz, R., Bergmann, B., and Goebel, W. (1999) Mol. Microbiol. 31, 557-567
8. Oscarsson, J., Mizunoe, Y., Li, L., Lai, X. H., Wieslander, A., and Uhlin, B. E. (1999) Mol. Microbiol. 32, 1226-1238
9. Atkins, A., Wyborn, N. R., Wallace, A. J., Stillman, T. J., Black, L. K., Fielding, A. B., Hisakado, M., Artymiuk, P. J., and Green, J. (2000) J. Biol. Chem. 275, 41150-41155
10. Schagger, H., and von Jagow, G. (1991) Anal. Biochem. 199, 223-231
11. van Heel, M., and Keegstra, W. (1981) Ultramicroscopy 7, 113-130
12. van Heel, M., Harauz, G., Orlova, E. V., Schmidt, R., and Schatz, M. (1996) J. Struct. Biol. 116, 17-24
13. Crowther, R. A., and Amos, L. A. (1971) J. Mol. Biol. 60, 123-130.
14. Henderson, R., Baldwin, J. M., Downing, K. H., Lepault, J., and Zemlin, F. (1986) *Ultramicroscopy* 19, 147-178
15. Crowther, R. A., Henderson, R., and Smith, J. M. (1996) *J. Struct. Biol.* 116, 9-16
16. Wai, S. N., Lindmark, B., Soederblom, T., Takade, A., Westermark, M., Oscarsson, J., Jass, J., Richter-Dahlfors, A., Mizunoe, Y., and Uhlin, B. E. (2003) *Cell* 115, 25-35
17. Radermacher, M., Wagenknecht, T., Verschoor, A., and Frank, J. (1987) *J. Microsc.* 146, 113-136
18. Miller, K. I., Schabtach, E., and Van Holde, K. E. (1990) *Proc. Natl. Acad. Sci. USA* 87, 1496-1500
19. Orlova, E. V., Dube, P., Harris, J. R., Beckman, E., Zemlin, F., Markl, J., and vanHeel, M. (1997) *J. Mol. Biol.* 271, 417-437
20. Tilley, S. J., Orlova, E. V., Gilbert, R. J. C., Andrew, P. W., and Saibil, H. R. (2005) *Cell* 121, 247-256
21. White, S. H., Ladokhin, A. S., Jayasinghe, S., and Hristova, K. (2001) *J. Biol. Chem.* 276, 32395-32398
22. Slade, A., Luh, J., Ho, S., and Yip, C. M. (2002) *J. Struct. Biol.* 137, 283-291
23. Parker, M. W., and Feil, S. C. (2005) *Prog. Biophys. Mol. Biol.* 88, 91-142
24. van Dam, L., Karlsson, G., and Edwards, K. (2004) *Biochim. Biophys. Acta* 1664, 241-256

**FOOTNOTES**

*This work was supported by BBSRC. N.C. was supported by the Socrates-Erasmus programme of the European Commission.

1Present address: Avecia Biotechnology, Belasis Avenue, Billingham, Cleveland TS23 1YN, UK

2Present address: Max-Planck-Institut für Molekulare Physiologie, Abteilung Physikalische Biochemie, Otto-Hahn-Str. 11, D-44227 Dortmund, Germany

3The abbreviations used are: 3D, three-dimensional; BTLE, brain total lipid extract; CMC, critical micelle concentration; EM, electron microscopy; HlyE, Hemolysin E (abbreviations ClyA or SheA are also used for this protein); MRA, multi-reference alignment; MSA, multivariate statistical analysis; β-OG, *n*-octyl-β-D-glucopyranoside; TCB, thrombin cleavage buffer.

**ACKNOWLEDGEMENTS**

We thank Dr. P. Wang for help and support with EM and Dr. A. J. G. Moir for the N-terminal sequencing.

**FIGURE LEGENDS**

FIGURE 1. *HlyE monomer and membrane-bound complexes.* *A*, Ribbon representation of the water-soluble monomer. *B*, Single pores and spherical aggregates of pores form on reconstitution of HlyE pores in lipid bilayers. *C*, Rarely, single pores in side views (arrows) can be found. Scale bars 50 nm.

FIGURE 2. *Electron microscopy of HlyE in n-octyl-β-D-glucopyranoside.* *A*, Micrograph of negatively stained HlyE pores. (1) Individual pores, and (2) small clusters in top view can be seen. (3) Some clusters lie on the carbon film with the pores on their sides. (4) Side views of individual HlyE pores are very rare. Scale bar 50 nm. *B*-D, Symmetry of the pores from the three size groups found. Rotational power spectra (13) of the top view total averages of "big" (B) "medium" (C) and "small" (D) pores. Insets: corresponding individual raw images and averages for each group.
FIGURE 3. Imaging of HlyE at different brain lipid/n-octyl-β-D-glucopyranoside ratios (w/w). A, Diagram and B, electron microscopy of the observed types of pore aggregates at different relative lipid and detergent concentrations. (1, ◆) Pores in lipid vesicles; (2, ○) few pores possibly inserted in bilayer fragments or in discoidal micelles (24); (3, ●) spherical aggregates of pores; (4, □) paracrystalline “sheets” and increased fraction of individual pores in side views; (5, △) single pores aggregating into “chains”; (6, ▲) single pores with a preponderance of top views. Symbols represent observations at certain relative concentrations and vary according to the type of the pore aggregates found. The pores in Fig. 5A were formed under conditions (4) and (5) (Fig. 5A, inset). Scale bar 50 nm.

FIGURE 4. Truncated pores formed by partial hydrolysis. A, Negatively stained truncated pores mixed with lipid vesicles. The pores do not appear to insert into the lipid. Scale bar 50 nm. B, Rotational average of the total sum of 102 top views of truncated pores (cf. Fig. 2C-D). C, Total average of 161 individual side view images of truncated pores (cf. Fig. 5C, D). Threshold for the measurements is at the steepest gradient between the densities of the pore and the surrounding stain. D, Mass-spec and sequencing data indicate that truncated pores are complexes built from two HlyE fragments containing the whole of αB, part of αC, part of αE and the whole of αG. Sequences not confirmed in the truncated pores are represented as Ca-wire in this diagram of the water soluble HlyE monomer (cf. Fig. 1A).

FIGURE 5. Electron microscopy of HlyE in brain total lipids/n-octyl-β-D-glucopyranoside (see also Fig. 3). A, Negatively stained HlyE pores in mixed micelles. (1) There are some side views of individual pores, but most of the pores in side views are clustered in small groups of two (2) or three (3). (4) The majority of the pores are in top (end) views. (5) Inset: under certain conditions (see Fig. 3 for details) long "chain-like" aggregates of pores are formed. In all side-view clusters, the pores are connected at one of their ends, only. Scale bar 50 nm. B, Representative individual images, C, corresponding averages and D, reprojections orthogonal to pore axis of the final three classes. From left to right: "squat" pores, "wide" pores and "narrow" pores. The association end of each pore is at the bottom. Scale bar 10 nm. E, Fourier shell correlation for the three-dimensional reconstructions. "Squat" pores (⋯⋯), "wide" pores (— —) and "narrow" pores (——). 

FIGURE 6. HlyE pore three-dimensional (3D) reconstructions: (1) "squat", (2) "wide" and (3) "narrow" pores. A, Dimensions of the molecular envelopes of the final three reconstructions before flattening correction. The association end is at the bottom. The three surface views are generated by restricting the resolution at 50 Å and rotationally averaging of the corresponding reconstruction, followed by surface rendering at the maximum density gradient. B, Comparison between the symmetrised averages from Fig. 2C-D and top view reconstructions (rescaled by 0.9) from the three reconstructions. In each image, the left half is from the symmetrised top view average, the right half is from the reprojection. Views of the "big" top view averages are compared with reprojections of the "squat" pores (1) and the "wide" pores (2). Views of the "medium" top view averages are compared with reprojections of the "narrow" pores (3). Noise features on the symmetry axis are reinforced by the symmetry averaging. C, Surface views of the reconstructions filtered at their resolution limit (Fig. 5E). Volumes are corrected for flattening, and noise along the symmetry axis has been masked. The threshold for surface rendering is at the steepest 3D density gradient. Two subunits have been removed for clarity. The association end is at the bottom. Scale bar 5 nm. D, Docking of the X-ray structure to a three-dimensional (3D) map of the "narrow" HlyE pore. X-ray crystal structure of the water soluble monomer is placed within the map with the "head" domain towards the association/putative membrane end of the reconstruction, as suggested from the analysis of the truncated pores. The 3D map is generated by low-pass filtering of the "narrow" pore reconstruction at 50 Å, and is represented by a mesh drawn at the same threshold level used to render the surface in Fig. 6A, image 3. The resolution is insufficient to reveal structural changes that might take
place; the figure merely illustrates the need for a structural change to accommodate the increased length of the subunits within the pore assembly.
Structure of the hemolysin E (HlyE, ClyA, SheA) channel in its membrane-bound form
Svetomir B. Tzokov, Neil R. Wyborn, Timothy J. Stillman, Stuart Jamieson, Nadine Czudnochowski, Peter J. Artymiuk, Jeffrey Green and Per A. Bullough

J. Biol. Chem. published online June 5, 2006

Access the most updated version of this article at doi: 10.1074/jbc.M602421200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts