The formation and structure of *Escherichia coli* K-12 haemolysin E pores

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Some enteric bacteria synthesize a pore-forming toxin, HlyE, which is cytolytic and cytotoxic to host cells. Measurement of HlyE binding to erythrocyte ghosts and the kinetics of HlyE-mediated erythrocyte lysis suggests that interaction with target membranes is not the rate-limiting step in the formation of HlyE pores, but that there is a temperature-dependent lag phase before a functional pore is formed. Circular dichroism and fluorescence energy transfer analyses show that HlyE protomers retain an α -helical structure when oligomerized to form a pore consisting of parallel HlyE protomers. Comparison of the proteolytic sensitivities of the water-soluble and oligomeric forms of HlyE identifies inner and outer surfaces of the pore. This new information has been used to constrain a model of the HlyE pore, which allows a more detailed interpretation of previous low-resolution 3D reconstructions and suggests a novel mechanism for insertion of HlyE into target membranes.

INTRODUCTION

Many pathogenic bacteria produce toxins to disrupt host-cell functions. Haemolysin E (HlyE; also known as ClyA or SheA) is a pore-forming toxin synthesized by *Escherichia coli* and other enteric bacteria (del Castillo *et al.*, 1997; Green & Baldwin, 1997; Ludwig *et al.*, 1999, 2004; Oscarsson *et al.*, 1996, 2002). HlyE lyses mammalian erythrocytes, is cytotoxic toward cultured mammalian cells, and induces apoptosis in macrophages (Lai *et al.*, 2000). Recently, it has been shown that HlyE antibodies are present in humans that have been infected with either *Salmonella enterica* serovar Typhi or serovar Paratyphi A, suggesting that HlyE is involved in pathogenesis (von Rhein *et al.*, 2006). Furthermore, the *hlyE* gene is present in 45 % of extraintestinal *E. coli* clinical isolates (Kerenyi *et al.*, 2005). Regulation of *hlyE* expression is complex and is influenced by several environmental signals. A single site in the *hlyE* promoter enhances expression in response to oxygen starvation when occupied by FNR (regulator of fumarate nitrate reduction) (Green & Baldwin, 1997; Wyborn *et al.*, 2004b) and in response to glucose starvation when occupied by CRP (cAMP receptor protein) (Westermarck *et al.*, 2000; Wyborn *et al.*, 2004b). In addition, the nucleoid-structuring protein H-NS has a negative effect on FNR-driven *hlyE* expression and a

positive effect on CRP-driven *hlyE* expression (Westermarck *et al.*, 2000; Wyborn *et al.*, 2004b). Furthermore, it is thought that H-NS-mediated repression is antagonized by a fourth transcription factor, SlyA (Wyborn *et al.*, 2004b).

The 3D structure of the water-soluble form of HlyE shows that it is a 34 kDa rod-shaped molecule consisting of a bundle of four long [80–90 Å (8–9 nm)] helices, which coil around each other with significant elaborations at both poles of the four-helix bundle (Fig. 1; Wallace *et al.*, 2000). At the end containing the N- and C-terminal regions of the protein a shorter [30 Å (3 nm)] helix (α G) packs against the four long helices, forming a five-helix bundle for about one-third of the length of the molecule (the tail domain). Random and site-directed mutagenesis has revealed that residues in the α G region play important roles in HlyE activity (Atkins *et al.*, 2000; Oscarsson *et al.*, 1999). HlyE possesses only two cysteine residues and these are housed in the tail domain (Fig. 1). It has been reported that the redox state of the protein (dithiol, in the cytoplasm and outer-membrane vesicles, or disulphide, in the periplasm) affects the oligomeric state of HlyE (Atkins *et al.*, 2000; Wai *et al.*, 2003), but more recently both reduced and oxidized HlyE has been shown to be active (Eifler *et al.*, 2006). At the other end of the rod there is a subdomain (the head domain) that consists of a short two-stranded hydrophobic antiparallel β -sheet flanked by two short helices (the β -tongue) (Fig. 1a; Wallace *et al.*, 2000). Site-directed mutagenesis has shown that the hydrophobic nature of the β -tongue has to be maintained to allow HlyE to bind to and lyse target cells (Oscarsson *et al.*, 1999; Wallace *et al.*, 2000). In the crystal structure, HlyE is a head-to-tail dimer

Abbreviation: β -OG, *n*-octyl β -D-glucopyranoside.

Two supplementary figures of models of octameric and 13-meric HlyE pores showing proteolytic cleavage sites and of the locations of proteolytic sites mapped onto an HlyE protomer are available with the online version of this paper.

Fig. 1. 3D structure of HlyE. (a) The HlyE monomer; the HlyE protein main chain is shown. The locations of the N and C termini, α G, the two Cys residues (black circles), the four-helix bundle, the β -tongue and the head and tail domains are indicated. (b) The structure of the HlyE dimer. The two HlyE monomers form a head-to-tail dimer across the twofold axis. The hydrophobic β -tongue of one monomer packs against a hydrophobic surface, formed from residues at the C-terminal end of α B and neighbouring side chains in α C and α G, of the other monomer. The individual monomers are shown in dark and light grey.

in which the hydrophobic β -tongue of one protomer packs against a second hydrophobic surface in the tail domain of the second protomer (Fig. 1b; Wallace *et al.*, 2000). Furthermore, gel filtration shows that in solution *E. coli* K-12 HlyE exists as a mixture of monomers, dimers and higher-order aggregates (Atkins *et al.*, 2000), and that HlyE from an avian pathogenic *E. coli* is predominantly dimeric (Wyborn *et al.*, 2004a). Thus, it would appear that the oligomeric state of HlyE in solution is dynamic and that the monomeric form of the protein is perhaps the form most likely to form initial interactions with a target membrane, because the hydrophobic β -tongue is exposed.

HlyE disrupts host cells by forming pores in target membranes (Wallace *et al.*, 2000). Initial electron micrographs of the pores suggested that HlyE does not undergo large conformational changes during pore formation (Wallace *et al.*, 2000). However, two recent descriptions of 3D reconstructions have revealed that the HlyE pores are longer than the water-soluble form of the protein, indicating that significant conformational changes are necessary to form a functional pore (Eifler *et al.*, 2006; Tzokov *et al.*, 2006). Although both reconstructions were generated from very similar objects, the interpretation of the data led to the conclusion that the HlyE pore was predominantly octameric in one case (Tzokov *et al.*, 2006) and predominantly 13-meric in the other (Eifler *et al.*, 2006). The reason for the discrepancy is unclear.

Eifler *et al.* (2006) proposed a model of pore formation in which membrane-bound HlyE monomers undergo a rate-limiting conformational change that precedes oligomerization

to form a pore. We report here an analysis of the formation and structure of the HlyE pore that shows: (i) interaction with target membranes is not the rate-limiting step in the formation of HlyE pores; (ii) HlyE protomers retain a mostly α -helical structure when oligomerized to form a pore; (iii) HlyE protomers in the oligomer are parallel. In addition, we have used partial proteolysis of the water-soluble and oligomeric forms of HlyE to identify the inner and outer surfaces of the HlyE pore. This information was used to constrain a model of an octameric assembly of HlyE protomers, allowing a more detailed interpretation of the 3D reconstructions of the HlyE pore obtained by electron microscopy.

METHODS

Haemolysin assay. HlyE activity was measured essentially as described by Rowe & Welch (1994). Briefly, HlyE protein (isolated as previously described; Atkins *et al.*, 2000) was incubated with defibrinated sterile horse blood (TCS Microbiology) in Tris-buffered saline (50 mM Tris/HCl, pH 8.0, containing 150 mM NaCl). The amount of haemolysis was determined spectrophotometrically by measuring haemoglobin release at 543 nm in cell-free supernatants prepared by centrifugation of reactions at 3000 r.p.m. in a bench top microcentrifuge for 30 s. Total haemolysis (100%) was defined by incubation of red blood cells in water, in place of buffer, and measuring A_{543} of the supernatant. The effects of *n*-octyl β -D-glucopyranoside (β -OG; Roche) on HlyE activity were determined by measuring the haemolytic activity of samples of HlyE protein (1 μ M final concentration) after incubation at 20 °C for 30 min in the presence of the indicated concentrations of β -OG.

Measurement of haemolysis kinetics. Erythrocyte suspension (400 μ l of $\sim 1 \times 10^8$ cells ml^{-1}) in Tris-buffered saline was placed in a fluorimetry cuvette. The contents were equilibrated to the indicated temperatures and the reaction was initiated by the addition of HlyE (10 μ l; final concentration 2.5 μ M). Haemolysis was monitored in a Cary Eclipse Fluorescence Spectrophotometer set at 590 nm for both excitation and detection. Each assay was repeated at least twice. Activation energies for the lag and haemolytic phases were calculated as described by Harris *et al.* (1991).

Western blotting. The amount of HlyE bound to horse erythrocytes was estimated by Western blotting. After incubation of HlyE protein (12 nM, 600 ng) with horse red blood cells (2×10^9 cells in 1.5 ml, measured by counting the number of red blood cells using a haemocytometer and suitable dilutions of the red blood cell suspension) for the indicated times, the reactions were centrifuged (see above) to isolate supernatant and membrane fractions. The membrane fraction was washed once with 1 ml Tris-buffered saline and then solubilized in loading buffer for SDS-PAGE (Sambrook & Russell, 2001). After determining the amount of haemolysis by measuring the A_{543} of the supernatants, aliquots were also prepared for electrophoresis by mixing with SDS-PAGE loading buffer. After electrophoresis, the proteins were transferred from the polyacrylamide gels (15%) to nitrocellulose (Hybond C extra, Amersham). The blots were probed with anti-HlyE serum (1:20 000, raised in rabbits) for 3 h, before washing and exposure to HRP-linked anti-rabbit IgG and detection by ECL-Plus (Amersham). The resulting films were analysed by quantitative densitometry using the Imagemaster software package (Amersham).

Circular dichroism spectroscopy. Far-UV circular dichroism spectra were recorded using a Jasco J-810 spectropolarimeter with

HlyE protein (400 nM) in 20 mM sodium phosphate buffer, pH 7.0. To observe the effects of oligomerization on HlyE secondary structure, the protein was pre-incubated for 30 min at 20 °C with β -OG (20 mM).

Electron microscopy. Negatively stained images of HlyE pores in β -OG were obtained essentially as described by Tzokov *et al.* (2006), except that the β -OG concentration used was 20 mM (1 $\times$ critical micelle concentration).

Fluorimetry. Aliquots of HlyE protein (6 μ M) were labelled by incubation at 4 °C for 16 h in the presence of a 10-fold molar excess of either isothiocyanate or maleimide derivatives of fluorescein or rhodamine (Molecular Probes) dissolved in dimethylformamide. Unreacted probe was quenched by addition of either lysine (1 mM) or DTT (1 mM), as appropriate. Labelled protein was isolated by size exclusion chromatography using Hi-Trap desalting columns (GE Healthcare) equilibrated with Tris-buffered saline, and the extent of protein labelling was estimated spectrophotometrically using molar absorption coefficients of 83 000 M⁻¹ cm⁻¹ for fluorescein and 95 000 M⁻¹ cm⁻¹ for rhodamine. The HlyE proteins (~0.2 mg ml⁻¹) labelled at Lys residues carried an average of 3.3 labels per molecule, whereas the Cys-labelled proteins carried 0.4 labels per HlyE. The haemolytic activities of the labelled proteins were similar to that of unlabelled HlyE, which had been similarly treated with dimethylformamide only, except for HlyE labelled at Lys residues with fluorescein, which retained 30 % of the activity observed with the unlabelled protein. In all experiments the ratio of donor molecules to acceptor molecules based on protein concentration was 1:7. Erythrocyte ghosts were prepared by adding 2 ml defibrinated horse blood (~10¹⁰ cells) to 100 ml 5 mM phosphate buffer, pH 7.5 containing 1 mM EDTA to lyse the red blood cells. The membrane fraction was separated from the soluble fraction by centrifugation for 20 min at ~20 000 g at 4 °C. After repeated washing, haemoglobin-free membranes were collected by centrifugation. The membranes were suspended in 2 ml 10 mM phosphate buffer, pH 7.5 containing 5 mM MgCl₂ and partially resealed by incubation at 37 °C for 30 min. Erythrocyte ghosts were added to a final concentration equivalent to ~5 $\times$ 10⁷ erythrocytes ml⁻¹. Fluorescence energy transfer was measured by exciting the fluorescein-labelled donor molecules at 470 nm and recording the emission spectra from 500 to 600 nm using a Cary Eclipse Fluorescence Spectrophotometer (Varian).

For intrinsic tryptophan fluorescence measurements the excitation wavelength was 280 nm and emission spectra were collected at 300–480 nm using a Cary Eclipse Fluorescence Spectrophotometer (Varian).

Partial proteolysis of HlyE. Aliquots of HlyE (35 μ g) in PBS, in the presence or absence of 50 mM β -OG, were proteolytically digested with trypsin, α -chymotrypsin, Asp-N or V8 protease, with protease to protein ratios of 1:20 at 37 °C. Samples were taken hourly and either immediately frozen at -20 °C for analysis by MS or mixed with SDS-PAGE loading buffer (Sambrook & Russell, 2001) and heated at 95 °C for 10 min for analysis by SDS-PAGE. In the latter case, after electrophoresis the polypeptides were blotted onto a PVDF membrane and excised for analysis by N-terminal sequencing (Procise 392, ABI). Alternatively, samples were analysed by MS using an ABI Voyager-DE STR MALDI mass spectrometer operating in positive-ion mode. Peptide analysis was performed in reflector mode using α -cyano-4-hydroxy-cinnamic acid (Sigma) as matrix; sinapinic acid (ABI and Fluka) was used as the matrix for protein analysis. Both were prepared immediately before use at a concentration of 10 mg ml⁻¹ in 50 % acetonitrile containing 0.05 % trifluoroacetic acid. Typically, peptide and protein samples were diluted three- to fivefold with matrix and 1 μ l was spotted onto the MALDI plate. The

spectrometer was calibrated using commercially available calibration standards (ABI and Sigma).

Model building. Models of HlyE multimeric assemblies were created by application of symmetry to the crystallographic coordinates of the monomer (Wallace *et al.*, 2000). The centre of gravity of the monomer was placed on the origin and its major axis aligned with the z axis of the coordinate system. The distribution of protected and unprotected cleavage sites on monomeric coordinates was observed, and the monomer was translated orthogonally from the z axis so as to maximize the number of unprotected proteolytic cleavage sites on the outside of the model of the multimer. A translation of ~40 Å (~4 nm) was used to give agreement with the electron microscopic observations of the size of the pore (Eifler *et al.*, 2006; Tzokov *et al.*, 2006). Symmetric pore models were then created by application of *n*-fold symmetry around the z axis (where *n*=7–13), and improved by rigid-body rotations of the initial monomeric model.

RESULTS

Binding of HlyE to target cells

After recognizing a membrane target, HlyE must switch from a water-soluble dimer (Wallace *et al.*, 2000; Wyborn *et al.*, 2004a) to a membrane-bound oligomer. To investigate the kinetics of this switch, horse blood (2 $\times$ 10⁹ cells in 1.5 ml) was incubated with purified HlyE (12 nM; 600 ng) at 15 and 37 °C, and samples (0.15 ml) were withdrawn at the indicated times (Fig. 2a). Membrane-bound HlyE was estimated by Western blotting and red blood cell haemolysis was measured. This revealed that although the extent of haemolysis was much greater at 37 °C than at 15 °C and cell lysis continued to increase up to 180 min, the binding of HlyE at both temperatures was maximal within 5 min (the first sample point in these experiments) (Fig. 2a). In other experiments the amount of HlyE remaining in solution was found to be similar after incubation with red blood cells for 1, 2, 5 and 10 min at 17, 22, 27, 32 and 37 °C (not shown). Furthermore, pre-incubation of HlyE (12 nM) with erythrocytes (2 $\times$ 10⁸ cells) at 15 °C for 20 min to allow HlyE binding with minimum cell lysis (8.9 $\pm$ 0.7 % lysis) did not enhance cell lysis upon transfer to 37 °C for 15 min (82 $\pm$ 0.3 % lysis) compared to control reactions that were not pre-incubated at 15 °C, but held at 37 °C for 15 min (70 $\pm$ 5.5 % lysis). Thus, it was concluded that the binding of HlyE to a target membrane is not the rate-limiting step in pore formation.

Incubation of HlyE for 30 min at 20 °C in the presence of different concentrations of the detergent β -OG resulted in inhibition of HlyE activity (Fig. 2b), and induced the formation of HlyE pores that resembled those formed in lipid vesicles (Fig. 2c). Incubation of HlyE with β -OG (20 mM) resulted in the protein eluting in the void volume when subjected to gel filtration on Sepharose 6B, indicating a molecular mass of ~500 000 (results not shown). However, incubation with β -OG did not significantly alter the circular dichroism spectrum of HlyE, which was typical of a protein rich in α -helices, consistent with the crystal structure (Fig. 2d). Together, these results suggest that

Fig. 2. The effect of temperature on HlyE binding to and lysis of target cells and the effects of detergent on the secondary structure, oligomeric state and activity of HlyE. (a) A sample of HlyE (12 nM, 600 ng) was added to horse erythrocytes (2×10^9 cells in 1.5 ml) and incubated at 15 or 37 °C. The extent of haemolysis of the red blood cells (◆, 15 °C; ■, 37 °C) and the relative amounts of erythrocyte-associated HlyE, measured by Western blotting (◇, 15 °C; □, 37 °C) were estimated at the indicated times. (b) Inhibition of HlyE activity by pre-incubation at 20 °C for 30 min in the presence of the indicated concentrations of β -OG. HlyE activity was measured at 37 °C in reactions containing 1 μ M HlyE. An activity of 100% was defined as that obtained in the absence of β -OG. The points represent means $\pm$ SD ($n=3$). (c) Electron micrograph of HlyE after incubation with β -OG. The micrograph shows negatively stained HlyE oligomers after incubation with β -OG (20 mM) at 20 °C for 30 min. Top views of HlyE pores (arrowed) with similar dimensions to those observed in lipid vesicles are visible. (d) Circular dichroism spectra of HlyE protein (400 nM) in 20 mM sodium phosphate buffer, pH 7.0, in the absence (solid line) and presence (dashed line) of β -OG (20 mM, pre-incubated at 20 °C for 30 min). (e) Intrinsic tryptophan fluorescence spectra of HlyE protein (600 nM) in 20 mM sodium phosphate buffer, pH 7.0, in the absence (lower line) and presence (upper line) of 20 mM β -OG.

HlyE oligomerization is not accompanied by major changes in the α -helical content of the protein. However, this does not mean that there are no significant structural rearrangements upon pore formation. For example, the circular dichroism spectra of pneumolysin show relatively small changes in the presence or absence of cholesterol (Kelly & Jedrzejewski, 2000), but there are major rearrangements of pneumolysin protein domains during the process of pore formation (Tilley *et al.*, 2005).

The HlyE protein has two Trp residues (Trp37 and Trp86). In the water-soluble form these Trp residues are located close together approximately at the mid-point of the HlyE rod. Intrinsic tryptophan emission spectra of HlyE exhibited an emission maximum at 334 nm, suggesting that both Trp residues are essentially solvent-shielded (Fig. 2e). This is consistent with the crystal structure, which shows that only the edge of Trp37 is surface-exposed and

that Trp86 is buried. Detergent (20 mM β -OG)-triggered oligomerization of HlyE caused a significant increase in intrinsic tryptophan fluorescence without altering the emission maximum (Fig. 2e). This suggests that upon oligomerization there are conformational changes in which the HlyE Trp residues remain solvent-shielded but become less ordered, causing an enhancement in fluorescence.

Kinetics of HlyE-mediated red blood cell lysis

Measurement of the kinetics of HlyE-mediated lysis of red blood cells showed that there was a temperature-dependent lag phase before haemolysis was detected (Fig. 3a). By plotting the reciprocal of the length of the lag phase against the reciprocal of the temperature it was possible to obtain a value of 60.2 ± 2.7 kcal mol⁻¹ (251.9 ± 11.3 kJ mol⁻¹) for the activation energy of the events occurring during the lag phase. From the rates of haemolysis following the lag

Fig. 3. Kinetics of HlyE-mediated erythrocyte lysis at different temperatures. (a) Lysis of erythrocytes ($\sim 4 \times 10^7$ cells in Tris-buffered saline) was determined by the decrease in light scattering upon addition of HlyE (2.5 μ M final concentration) at the indicated temperatures. (b) Arrhenius plots of HlyE lag phase and haemolysis. The activation energy for the lag phase (■) was determined by plotting the reciprocal of the lag phase ($1/t_{\text{lag}}$) against the reciprocal of the temperature ($1/T$). The activation energy for haemolysis (◆) was determined by plotting the log of the initial rate (k_0) against the reciprocal of the temperature.

phases the activation energy for haemolysis was calculated to be 23.3 ± 0.5 kcal mol $^{-1}$ (97.5 ± 2.1 kJ mol $^{-1}$) (Fig. 3b).

Conformational changes in HlyE upon membrane binding

The HlyE monomer possesses 32 Lys residues that could potentially be labelled with isothiocyanate derivatives of the fluorescent dyes fluorescein and rhodamine. The emission spectrum of HlyE labelled with fluorescein (HlyE_{DK}) in the absence of an acceptor was enhanced in the presence of erythrocyte ghost membranes (Fig. 4a, compare spectra I and II). This suggests that upon interaction with a membrane, HlyE undergoes conformational changes that enhance HlyE_{DK} fluorescence. In the presence of membranes and an acceptor (HlyE labelled with rhodamine; HlyE_{AK}), HlyE_{DK} fluorescence was

quenched and a fluorescence energy transfer signal was apparent at 560–580 nm (Fig. 4a, spectrum III). However, a fluorescence energy transfer signal was observed in the absence of membrane (Fig. 4a, spectrum IV), suggesting that, at least in part, the fluorescence energy transfer is due to exchange of protomers between HlyE dimers. Therefore, a second experiment, using fluorescent labels attached to the Cys residues of HlyE, was done.

The HlyE protein contains two Cys residues that are capable of forming an intramolecular disulphide bond (Atkins *et al.*, 2000). These Cys residues are located at the opposite pole (the tail region; Fig. 1) of the protein to that which interacts with target membranes (Atkins *et al.*, 2000; Eifler *et al.*, 2006; Tzokov *et al.*, 2006; Wallace *et al.*, 2000), and in the HlyE dimer the two pairs of cysteine residues are ~ 45 Å (4.5 nm) apart. The Cys thiol groups were labelled with maleimide derivatives of the fluorescent dyes fluorescein and rhodamine. The emission spectrum of HlyE labelled with fluorescein (HlyE_{DC}) in the absence of an acceptor was enhanced upon addition of erythrocyte ghost membranes, suggesting that the tail region of HlyE undergoes conformational changes that enhance HlyE_{DC} fluorescence (Fig. 4b, compare spectra I and II). In the presence of an acceptor (HlyE labelled with rhodamine; HlyE_{AC}) and a target membrane, HlyE_{DC} fluorescence was quenched and a fluorescence energy transfer signal was apparent at 560–580 nm (Fig. 4b, spectrum III). This fluorescence energy transfer signal was not observed in the absence of membrane (Fig. 4b, spectrum IV), suggesting that membrane-bound HlyE molecules are parallel, in contrast to the anti-parallel arrangement of the soluble dimer (Wallace *et al.*, 2000). All the responses described above were rapid, the reactions being complete within 30 s at temperatures in the 17–37 °C range. These observations suggest that HlyE dimer–monomer transitions, HlyE–membrane interactions and HlyE–HlyE interactions are not likely to be rate-limiting steps in target cell lysis.

Partial proteolysis of water-soluble and oligomeric HlyE, and construction of oligomeric pore models

To map regions that interact in the HlyE oligomer, the proteolytic digest patterns of water-soluble and β -OG (50 mM)-triggered HlyE oligomers were compared (Table 1). The identified cleavage points were plotted on the water-soluble HlyE structure, revealing regions that were protected from digestion in the HlyE oligomer (residues highlighted in purple, Fig. 5), and further regions that were accessible in both the water-soluble monomeric and dimeric forms and the oligomeric forms of the protein (residues highlighted in green, Fig. 5).

Recently, two 3D reconstructions based on analyses of electron micrographs of the HlyE pore have been described (Eifler *et al.*, 2006; Tzokov *et al.*, 2006). These analyses suggest that the HlyE pore is either predominantly octameric (Tzokov *et al.*, 2006), or predominantly 13-

Fig. 4. Fluorescence energy transfer between HlyE molecules in the presence of target membranes. (a) All samples contained HlyE (2.5 µg) labelled with fluorescein at Lys residues (HlyE_{DK}). Spectra were obtained in the absence of membranes (spectrum I), the presence of membranes (spectrum II), the presence of membranes and acceptor [HlyE, 10 µg, labelled with rhodamine at Lys residues (HlyE_{AK}) (spectrum III)] and the presence of acceptor without membranes (spectrum IV), at 37 °C in a total volume of 450 µl. The excitation wavelength was 470 nm. (b) As for (a), except that the donor was HlyE labelled with fluorescein at Cys residues (HlyE_{DC}), and the acceptor was HlyE labelled with rhodamine at Cys residues (HlyE_{AC}).

meric (Eifler *et al.*, 2006). Here, pore models constrained by partial proteolysis data were generated as described in Methods. Both octameric and nonameric models had

Table 1. Identification of sites sensitive to proteolysis in water-soluble and oligomeric HlyE

Aliquots of HlyE protein (1 nmol in 250 µl PBS) were incubated at 37 °C with trypsin, α-chymotrypsin, Asp-N or V8 protease. Samples (10 µl) were removed at intervals and analysed by SDS-PAGE and N-terminal amino acid sequencing or by MS to identify the peptide bonds in HlyE that are sensitive to proteolytic cleavage. Sites highlighted in bold type are unique to the indicated condition.

Site of proteolysis	
Water-soluble HlyE	Oligomeric HlyE
Glu3 (αA)	Glu3 (αA)
Asp7 (αA)	Asp7 (αA)
Glu11 (αA)	Glu11 (αA)
Lys14 (αA)	Lys14 (αA)
Glu18 (αA)	Asp21 (αA)
Asp25 (αA)	Asp25 (αA)
Asp32 (αA)	Glu46 (αA')
Glu46 (αA')	Glu258 (αF)
Glu53 (loop αA'–αB)	Glu276 (αG)
Glu106 (αC)	Glu287 (αG)
Lys107 (αC)	
Lys108 (αC)	
Asp114 (αC)	
Glu129 (αC)	
Asp171 (αD)	
Glu176 (αD)	
Glu256 (αF)	
Glu258 (αF)	
Glu276 (αG)	
Glu287 (αG)	

radial dimensions that agreed well with the electron microscopic observations (Eifler *et al.*, 2006; Tzokov *et al.*, 2006). However, because the water-soluble HlyE monomer is only 100 Å (10 nm) long, the length of the assembly is significantly shorter than the 140 Å (14 nm) observed in electron micrographs (Eifler *et al.*, 2006; Tzokov *et al.*, 2006). The octameric model yielded a lightly packed assembly of subunits (Fig. 5a–d), whereas there were a small number of steric clashes in the nonameric model, which became more severe in larger oligomers, unless the diameter of the model was increased. Thus, a 13-meric pore with similar interactions to the octameric model can be constructed, but requires a maximum diameter of more than 150 Å (15 nm; see Supplementary Fig. S1). This diameter is larger than that observed in electron micrographs, but possibly at the outer limit of the errors in size measurement. Therefore, because of its agreement with the data presented here, and with the symmetry analyses of HlyE electron micrographs by Tzokov *et al.* (2006), the octameric model was adopted (Fig. 5d).

The model places the majority of the sites cleaved in the oligomer on the outside of the assembly (Fig. 5). The exception to this is Glu258, which is situated at the beginning of the turn between αF and αG and is located on the inside 'upper rim' of the assembly (Fig. 5c, and see Supplementary Fig. S2). Examination of the octameric model in detail reveals that αG of each protomer leans towards the groove between αA and αF in the neighbouring protomer (Fig. 6a, e), positioning the C-terminal residues (which are only partially ordered in the crystal structure of the monomer) in the groove. This feature of the model is congruent with the deleterious effects on pore assembly of the deletion of αG and the C-terminal residues (Atkins *et al.*, 2000; Oscarsson *et al.*, 1999). It is also notable that the only proteolytic cleavage point that is seen in the multimer, but not in the monomer, is at Asp21 (Fig. 5c). Asp21 forms a hydrogen

Fig. 5. Model of the proposed octameric HlyE assembly showing proteolytic cleavage sites. Views are shown of a simple space-filling model of an octameric pore assembly illustrating the relative orientations of the HlyE protomers (a) from the side and (b) from above. (c) A slightly tilted view of the model with six of the protomers made semi-transparent to give a clear view of the remaining two, chosen to show the positions of the proteolytic cleavage points on the outside (left protomer) and inside (right protomer) of the assembly. (d) A view of the inside surface of the pore model with dimensions of the model indicated. (e) A cartoon view of the HlyE backbone indicating the spatial relationship between α G (light blue) and the C-terminal (red) of one protomer (pale brown) and the adjacent protomer (white). In all the representations, alternate protomers are coloured white and pale brown, and the 24-residue hydrophobic sequence that includes the β -tongue, which is required for membrane binding, is coloured dark grey. The C-terminal α G helix is coloured light blue. Proteolytic cleavage sites observed in both the water-soluble and oligomeric forms of HlyE are coloured green and labelled in black, cleavage sites protected in the oligomer are coloured and labelled in purple, and the residue (Asp21) that is sensitive in the oligomer, but not in the water-soluble form of HlyE, is coloured and labelled in dark blue. Diagrams were produced using PyMOL (DeLano & Lam, 2005).

bond with Thr284 in α G, possibly indicating a minor conformational change in this region, which may also account for the protection of Glu18, the only cleavage point on the outside of the model that is protected. The paucity of new cleavage sites in the oligomer compared to the water-soluble protein suggests that any conformational changes are likely to be limited.

DISCUSSION

HlyE-mediated cell lysis is the product of a complex series of steps in which HlyE must recognize and bind to the target host-cell membrane and then assemble to form a functional pore. Previous evidence suggests that water-soluble HlyE exists as a mixture of monomers and dimers (Atkins *et al.*, 2000; Wallace *et al.*, 2000; Wyborn *et al.*, 2004a). It is suggested that the HlyE dimer, in which the hydrophobic surfaces in the head (β -tongue) and tail (residues located in helices B, C and G) are shielded from the solvent, helps maintain HlyE in a water-soluble form. Thus, it is likely that the monomeric and dimeric forms of HlyE are in equilibrium, and the data presented here suggest that binding of HlyE monomers, with a solvent-exposed β -tongue, to a target membrane is fast. The fluorescence energy transfer experiments suggest that following interaction with the membrane, HlyE protomers

rapidly oligomerize. Thus, it is suggested that neither membrane binding nor initial interactions between membrane-bound HlyE monomers are rate-limiting steps in creating a functional pore. Nevertheless, during these rapid phases HlyE undergoes conformational changes in regions, including those (tail domain, Fig. 1) that are remote from the β -tongue, which are essential for interaction with a membrane (Wallace *et al.*, 2000; Oscarsson *et al.*, 1999). The changes in HlyE conformation are likely to be required for binding the membrane and for subsequent initial interactions between HlyE protomers to form parallel membrane-bound HlyE oligomers. This rapid phase is then followed by a slow component, most apparent as a temperature-dependent lag phase, with relatively high activation energy, before haemolysis occurs. Taken together, these observations indicate that whereas HlyE binding to a host-cell membrane and initial oligomerization are rapid, functional pore formation is a slower process. Such a mechanism accounts for the relatively poor haemolysis observed at 15 °C compared to 37 °C and the need for prolonged incubation for cell lysis to occur at low HlyE concentrations. In these respects, HlyE resembles the HlyA protein of *E. coli* (Eberspacher *et al.*, 1989). Thus, the data presented broadly support the conclusions of Eifler *et al.* (2006), who also suggest that there is a rate-limiting conformational transition in membrane-bound HlyE that precedes the formation of a functional pore. It is now

suggested that the slowly reacting monomeric intermediate proposed by Eifler *et al.* (2006) contains an oligomeric component.

Recently, two 3D reconstructions of the HlyE pore have been reported, which although they represent the same object, apparently contain different numbers of subunits (Eifler *et al.*, 2006; Tzokov *et al.*, 2006). The reasons for this discrepancy are not clear. Nevertheless, several features of the modelled HlyE pore shown in Fig. 5 are consistent with the 3D reconstructions (Eifler *et al.*, 2006; Tzokov *et al.*, 2006). Thus, in the simple model shown in Fig. 6 the hydrophobic β -tongue is outward facing, and thus has the potential to interact with the lipid tails of a target membrane bilayer. At the opposite end of the pore there is a region of ~ 35 Å (~ 3.5 nm) in length over which the channel wall is thicker (Eifler *et al.*, 2006; Tzokov *et al.*, 2006). The tail domain of HlyE occupies this region in the constrained model shown in Fig. 6, and the presence of the 35 Å (3.5 nm) α G helix that contributes to the five-helix bundle of the tail domain (Fig. 1) is consistent with the channel wall thickening in this region of the reconstructions (Eifler *et al.*, 2006; Tzokov *et al.*, 2006). Furthermore, the orientation of the HlyE subunits in Fig. 5 creates a negatively charged inner surface, consistent with zero-current membrane potential measurements (Ludwig *et al.*, 1999). The nature of the conformational changes that

result in the observed elongation of the HlyE pores remains unknown (Eifler *et al.*, 2006; Tzokov *et al.*, 2006), but the model using eight HlyE protomers shown in Fig. 5 exhibits plausible subunit packing with good matches to the pore dimensions obtained by electron microscopy, except for pore length. Thus, the length of the HlyE pore in the model is ~ 120 Å (~ 12 nm), compared to ~ 100 Å (~ 10 nm) for the water-soluble form of HlyE and ~ 140 Å (~ 14 nm) as measured from negatively stained and cryo-electron micrographs (Eifler *et al.*, 2006; Tzokov *et al.*, 2006; Wallace *et al.*, 2000). Therefore, some of the observed elongation of the HlyE molecule that occurs upon pore formation is accounted for in the model, and it is possible that some of the remaining difference is due to errors in the electron microscopy measurements.

It has been suggested that the β -tongue region of HlyE forms a 26-stranded β -barrel cap structure as part of the process of insertion into the membrane (Eifler *et al.*, 2006). However, it has not been suggested that this would constitute the final pore structure and indeed it is unlikely to do so. The β -tongue is composed almost entirely of hydrophobic residues, and a β -barrel transmembrane structure would result in an unprecedented pore structure with a hydrophobic lining as well as a hydrophobic membrane interface. This would be in stark contrast to the porin-like barrels of the majority of outer-membrane

Fig. 6. Model of an HlyE pore showing a putative membrane-spanning helical structure. (a) Structure of the head domain of HlyE with the hydrophobic residues in grey and the hydrophilic ones in orange; the hydrophobic putative transmembrane sequence including the β -tongue is on the left, the amphipathic (orange and grey) helix E is on the right; part of the main body of the protomer is in cyan. (b) Model in which the hydrophobic sequence becomes a single transmembrane helix (grey, left) and the polypeptide chain returns to the main body of the protomer via a realigned amphipathic helix E (orange and grey, right). (c) The hydrophilic face of helix E (shown entirely in orange for clarity) can then form the inner lining of a pore, while its hydrophobic face packs against the new transmembrane helix (grey) which interacts with lipid (shown schematically). (d) An octameric pore can be constructed based on the octameric α -helical barrel observed in Wza, shown in side view and looking down the eightfold axis (e). (f) An overview of the resulting model coloured as in Fig. 5 and Supplementary Figs S1 and S2.

proteins (Schulz, 2002; Parker & Feil, 2005), which have a hydrophilic lining and a hydrophobic outer face, made possible by their strand sequences which alternate between hydrophilic and hydrophobic residues. It is worthy of note that there is no such peptide sequence at any point in the HlyE sequence (Wallace *et al.*, 2000). Thus, Parker & Feil (2005) have argued that the transmembrane portion of HlyE is almost certainly helical. The position of the head domain in our pore model (Fig. 5) is potentially consistent with this. The head domain commences with the amphipathic α D helix, followed by a long hydrophobic sequence consisting of short helical segments and the β -tongue, which then rejoins the main body of the molecule (Fig. 1). It is an intriguing possibility that α D could form an octameric α -helical barrel pore, similar to that observed in the C terminus of *E. coli* Wza (Dong *et al.*, 2006), creating a hydrophilic pore of 20 Å (2 nm) diameter with the hydrophobic residues of α D facing outwards towards the membrane lipids (Fig. 6). This would be facilitated if the long hydrophobic sequence that includes the β -tongue, which has in fact been predicted to be a transmembrane helix (Oscarsson *et al.*, 1999), were to undergo a conformational change into an α -helix, which then returns to the original side of the membrane (Fig. 6a, b). This would increase the α -helical content of the molecule by only ~5 %, consistent with the circular dichroism data that suggest there is no large change in the helical content of HlyE upon pore formation. The new hydrophobic helix would make favourable interactions with the outward-facing side chains of the Wza-like α -helical barrel and with lipid (Fig. 6c, d). In order to make the required connections this helix would have to return at an angle of approximately 40° to the membrane perpendicular, which would be consistent with the extreme curvature of the membrane observed in electron micrographs of membranes and lipid vesicles treated with HlyE (Eifler *et al.*, 2006; Tzokov *et al.*, 2006; Wai *et al.*, 2003; Wallace *et al.*, 2000). Although the model is consistent with the available data it is speculative, and a definitive account of how and when the water-soluble HlyE structure becomes elongated, and the full details of the conformational changes that take place to enable the formation of the transmembrane channel, must await a high-resolution structure of the pore form of HlyE.

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