Structure-Function Relationships of a Novel Bacterial Toxin, Hemolysin E

THE ROLE OF αG*

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The novel pore-forming toxin hemolysin E (HlyE, ClyA, or SheA) consists of a long four-helix bundle with a subdomain (β tongue) that interacts with target membranes at one pole and an additional helix (αG) that, with the four long helices, forms a five-helix bundle (tail domain) at the other pole. Random amino acid substitutions that impair hemolytic activity were clustered mostly, but not exclusively, within the tail domain, specifically amino acids within, adjacent to, or interacting with αG. Deletion of amino acids downstream of αG did not affect activity, but deletions encompassing αG yielded insoluble and inactive proteins. In the periplasm Cys-285 (αG) is linked to Cys-87 (αG) of the four-helix bundle via an intramolecular disulfide. Oxidized HlyE did not form spontaneously in vitro but could be generated by addition of Cu(II) or mimicked by treatment with Hg(II) salts to yield inactive proteins. Such treatments did not affect binding to target membranes nor assembly into non-covalently linked octameric complexes once associated with a membrane. However, gel filtration analyses suggested that immobilizing αG inhibits oligomerization in solution. Thus once associated with a membrane, immobilizing αG inhibits HlyE activity at a late stage of pore formation, whereas in solution it prevents aggregation and consequent inactivation.

Disease-causing bacteria have adopted a number of strategies to inflict damage on host tissues in pursuit of their pathogenic lifestyles. Hemolysin E (HlyE) is a novel pore-forming toxin present in several pathogenic strains of Escherichia coli (including O157), Salmonella typhi, and Shigella flexneri and represents the first member of a new class of cytotoxin (1). In E. coli, hlyE expression is activated during anaerobic growth by the action of the global transcription factor FNR (2), and the product, HlyE, forms moderately cation-selective pores of 25–30 Å in diameter in target membranes (3, 4). The structure (Fig. 1b) of the soluble form of HlyE has recently been solved by x-ray crystallography (1). The HlyE monomer is 34 kDa and a rod-shaped molecule, consisting of a bundle of four long (80–90 Å) helices, which coil around each other with a simple up-down-up-down topology but with significant elaborations at both poles of the molecule (1). At the end of the bundle that contains the N-terminal region, an additional shorter (30 Å) helix (αG) packs against the four long helices, forming a five-helix bundle for about one-third of the length of the molecule (the tail domain). Site-directed mutagenesis has suggested that the αG region is multifunctional, involved in membrane targeting, pore formation, and translocation of HlyE (5). The tail domain also houses the two cysteine residues of HlyE, which are positioned close enough in adjacent helices to potentially form a disulfide bond (1). At the opposite end of the molecule there is a subdomain (the head domain) consisting of a short two-stranded antiparallel β sheet flanked by two short helices (the β tongue), located between the 3rd and 4th helices of the main bundle (1). The hydrophobic nature of the β tongue has to be maintained to allow interaction between HlyE and target membranes (1).

In EM studies, HlyE pores appear as ring-shaped assemblies with an internal diameter of 50–55 Å when viewed from above and as 105–100 Å spikes in side view, suggesting that HlyE does not undergo large conformational changes during pore formation (1).

Thus structural studies of HlyE have revealed the architecture of new family of bacterial toxins, but they have also raised a number of intriguing questions. Previous work has used site-directed mutagenesis to identify regions of HlyE required for activity (1, 5). Here a random mutagenic approach reveals that both poles of the protein are crucial for conferring a hemolytic phenotype on E. coli. These observations complement previous site-directed mutagenic studies in confirming the role of αG in HlyE activity, but reveal a new region at the opposite pole of the protein that is also required. Evidence is presented that during export an intramolecular disulfide is incorporated into HlyE linking αG to the rest of the tail domain. Incorporation of the disulfide, or bridging Cys-87 (αM) and Cys-285 (αG) with Hg(II), severely inhibits HlyE activity, indicating that upon release from the bacteria HlyE has to be reduced to become active. Evidence is presented to suggest that the assembled pore is an octameric assembly of HlyE subunits and that the disulfide bond may be introduced into HlyE to prevent premature oligomerization and consequent inactivation.

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1 The abbreviations used are: HlyE, hemolysin E; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; PCMBS, 4-(chloromercuri)-benzenesulfonic acid.

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**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Plasmids—** E. coli JM109 was used as the host for the *gst-hlyE* expression plasmid, pGS1111, that was constructed by ligating the PCR-mplified *hlyE* gene into pGEX-KG (6) utilizing engineered NcoI and SalI sites. *E. coli* MC1000 was the host for pGS1148, a ptac85 derivative containing the NcoI-SalI *hlyE*-coding region from pGS1111. The Altered Sites in *vitro* mutagenesis system (Promega) was used to introduce specific codon changes in *hlyE*. The substitutions made were C87S and C285S. Mutated *hlyE* genes were cloned into pGEX-KG to facilitate purification of the variants (C87S, pGS1269; C285S, pGS1268). Equivalent ptac85 derivatives were created to assess the activity of the *hlyE* variants in *vivo* (C87S, pGS1270; C285S, pGS1267) with MC1000 as the host. Mutant *hlyE* genes encoding variants with C-terminal deletions were created using PCR and cloned into ptac85 for the analysis of hemolytic phenotypes and activity measurements. A derivative of pQE31 (Qiagen) expressing an N-terminal His-tagged α helix was also constructed.

**Purification of HlyE—** Cultures of *E. coli* JM109 pGS1111 were grown aerobically at 37 °C in L-broth for 3 h before induction of *gst-hlyE* expression by addition of isopropyl-1-thio-β-D-galactopyranoside (100 μM ml⁻¹). After a further 3 h the bacteria were collected by centrifugation. Clarified French press cell extracts (up to 150 mg of total protein in 10 ml Tris-Cl, pH 8.0, containing 10 mg benzamidine and 0.1 mM phenylmethylsulfonyl fluoride) were applied to a column (50 × 15 mm) of GSH-Sepharose (Amersham Pharmacia Biotech) equilibrated with 25 mM Tris-Cl, pH 6.8, containing 150 mM NaCl and 2.5 mM CaCl₂. After washing with 10 volumes of the same buffer, the HlyE protein was released by thrombin treatment (5 units for 16 h at 25 °C). Protein concentrations were determined with the Bio-Rad protein reagent. The quality of the preparations used is illustrated in Fig. 2. HlyE crystallization, data collection, and analysis were as described (1). To obtain the disulfide form of HlyE, crystals were soaked in stabilization solution (1) containing 1 mM CuCl₂ for 9 days.

**Characterization of HlyE—** The hemolysis assay of Rowe and Welch (7) with horse blood was used to measure routinely the HlyE activity. Blood samples were obtained from TCS Microbiology (UK). The number of red blood cells per assay was determined using a counting chamber. Total metal ion contents and electrospray mass spectrometry measurements were done with samples of HlyE that had been diazylated against 1 mM ammonium acetate, pH 6.0. Native molecular weights were estimated by gel filtration on a calibrated Sepharose 6B column (370 × 16 mm) equilibrated with 25 mM Tris-Cl, pH 6.8. The existence of HlyE oligomers was confirmed by Western blotting using polyclonal anti-HlyE rabbit serum and an alkaline phosphatase anti-rabbit IgG conjugate or by ECL (Amersham Pharmacia Biotech). The blots were standardized using Bio-Rad pre-stained markers. Reaction thiols were determined colorimetrically by reaction of native protein with 5,5’-dithiobis(2-nitrobenzoic acid). Association of HlyE with target membranes was investigated by incubating HlyE, or Hg(ClI)-treated HlyE, with red blood cells and collecting the erythrocyte membranes by centrifugation. After washing the pelleted membranes (three times with HA buffer (7) and three times with 20 mM sodium phosphate, pH 6.5), reducing SDS-PAGE loading buffer was added, and Western blotting was then used to detect any HlyE protein that was associated with the erythrocytes. In further experiments the washed erythrocyte membranes were exposed to the chemical cross-linking reagent, dimethyl suberimidate (final concentration 0.5 mg ml⁻¹), for 4 h at 25 °C before analysis by SDS-PAGE and Western blotting. Native HlyE and Hg(ClI)-treated HlyE (0.2 mM Hg(II)) were used in these experiments. Western blotting was also used to detect exported HlyE in aliquots of culture medium treated with N-ethylmaleimide (5 mM for 30 min at 25 °C) to block any reactive thiols and to analyze HlyE in the *E. coli* periplasm. Periplasmic fractions from MC1000 ptac85 and MC1000 pGS1148 were obtained using the method of Duche et al. (8) and the presence (as judged by Western blotting) of the transcription factor FNR in the cytoplasmic but not in the periplasmic fraction was confirmed by using the integrity of the latter fraction.

**Error-prone PCR—** Random mutations in the *hlyE* gene of pGS1111 were introduced using Taq DNA polymerase and the following synthetic primers: 5′-GAGGGCAATGACCATGGCTGAATCAT (hlyE coordinates 135–160) and 5′-CAACAGTAACTGTGACCTTTTTA-ACCGT (1150–1181), where the unique NcoI and SalI targets are underlined. The PCR products were digested with NcoI and SalI before ligation between the corresponding sites in ptac85 (9). Plasmids were isolated by standard methods, and ABI cycle sequencing was used to identify mutations in the *hlyE* gene.

The library of mutagenized *hlyE* genes in ptac85 was used to transform *E. coli* strain DH5α. Transformants were screened for hemolysis on blood agar (5% v/v horse blood) containing 200 μg ml⁻¹ ampicillin. Colonies with reduced hemolysis were selected, purified, and grown in liquid culture (L-broth supplemented with ampicillin, 200 μg ml⁻¹) at 37 °C, and hemolytic activity was estimated in the soluble fraction of cell-free extracts. Western blotting with polyclonal anti-HlyE serum was used to confirm that the HlyE variants were both expressed and soluble.

**RESULTS**

**Overproduction and Properties of HlyE—** The overproduced glutathione S-transferase fusion protein, GST-HlyE, formed up to 27% of soluble cell protein yielding 2 mg of HlyE per liter of culture. Overproduced HlyE had a subunit molecular mass of 22 kDa and was estimated to be ≥95% pure as judged by SDS-polyacrylamide gel electrophoresis (Fig. 2). Because of the position of the thrombin cleavage site and the engineered NcoI site overlapping the initiating ATG codon, the released HlyE protein has an additional 15 N-terminal amino acids and a T2A substitution. The N-terminal amino acid sequence (GSPGIS-GGGLGDSMAEIVADKTV) confirmed the presence of the...
additional amino acids (underlined), the T2A substitution (bold), and that the purified protein was HlyE. Thus a convenient, efficient, high yielding procedure has been developed that is a significant improvement upon the previously published protocols (4, 5).

The mass of HlyE by electrospray mass spectrometry was exactly that predicted, indicating the absence of any post-translational modification. Optical spectra were featureless, except for a maximum at 279 nm (ε279 15,900 mm−1 cm−1). The thiol groups of both HlyE cysteine residues reacted readily with 5,5’-dithiobis(2-nitrobenzoic acid), and ion contents and electrospray mass spectrometry revealed that HlyE was metal-ion free (Table I). Thus, no evidence for a cofactor in the isolated HlyE protein was apparent from these investigations.

HlyE appears to be a robust protein as its activity was unaffected by incubation at 25 °C for 5 min at pH values between 3.0 and 9.0 before restoring the pH to 7.0. Also, t1/2 of HlyE was 1.5 min at 55 °C, and incubation with proteases (up to 2 units of trypsin, V8 protease, pepsin with 50 μg of HlyE) for 30 min at 37 °C failed to detectably degrade HlyE.

HlyE has a broad target spectrum displaying similar activities against human, rabbit, sheep, chicken, and horse erythrocytes in the standard assay. Upon dilution its specific activity remained constant until, at 2 nm, hemolytic activity became undetectable even after prolonged (16 h) incubation at 37 °C. This is consistent with previous observations that suggest a threshold concentration of HlyE has to be exceeded to detect hemolysis (10). By comparing the number of red blood cells in a standard assay with the concentration of HlyE added, it was estimated that 1000–2000 HlyE molecules are required to lyse an erythrocyte. This value compares well with 500 molecules for Vibrio cholerae cytolytin (11) but is much higher than the 5–20 molecules of E. coli HlyA (12).

Identification of Regions of HlyE Required for Activity—One approach to identify amino acids required for HlyE activity is to compare hlyE genes and identify highly conserved regions. The hlyE genes of 25 clinical isolates of E. coli that were judged to be more hemolytic on blood agar anaerobically than aerobically were sequenced. In all cases some part(s) of the coding regions were deleted such that a full-length HlyE protein could not be expressed. Thus, this sample was not useful in assigning conserved regions of the HlyE protein. Therefore, a library of randomly mutated hlyE genes was screened for those that reduce the degree of hemolysis on blood agar. This yielded 10 variants with less than 50% of the wild-type hemolytic activity in cell-free extracts (Table II). All were present in the soluble fractions of cell-free extracts indicating that the overall fold of the protein had not been distorted by the substitutions. The positions of substitutions causing defects in activity when superimposed on the three-dimensional structure of HlyE fell into several distinct regions (Fig. 1b). Five of the 10 variants were located in the tail domain (Fig. 1). Two of the variants had substitutions (R261K and Y263H) of amino acids with exposed side chains in the loop between αG and αO, and two others (K275R and K280E) were located in αO itself. The final activity compromising substitution in the tail domain (Y97H) was located in αO of the main four-helix bundle and disrupts a long range van der Waals interaction with Leu-274 (αQ) and a water-mediated H bond with Tyr-266 (αQ). It should be noted that the variant with the substitution K275R also contained a G180V replacement and that this latter change may also affect HlyE activity (Table II).

Previous studies have shown that deletion of 11 or 23 amino acids from the C terminus of HlyE severely compromises HlyE activity (5). To determine if the region downstream of αQ is required for HlyE activity, systematic deletions were made to create HlyE proteins lacking 2, 4, 6, 8, 12, and 37 C-terminal amino acids, respectively. These constructs were chosen on the basis of the structure to delete amino acids up to the C terminus of αQ (12 residue deletion) and to remove αQ entirely (37 amino acid deletion). Removing up to eight amino acids did not significantly impair HlyE activity in vivo (hemolysis on blood agar) or in vitro (HlyE activity in soluble cell-free extracts). However, E. coli strains expressing HlyE variants lacking 12 or 37 amino acids were non-hemolytic on blood agar and cell-free extracts lacked hemolytic activity. Western blotting indicated that these HlyE variants were probably misfolded, being well expressed but only present in the insoluble fraction of cell-free extracts, and thus could not provide any useful insight into HlyE structure-function relationships. Whether protein insolubility may have contributed to the severe inhibition caused by the C-terminal deletions reported by Oscarsson et al. (5) is not clear because it is not obvious whether the proteins were soluble, or insoluble, only that they were expressed. Expression of His-tagged αQ did not confer a hemolytic phenotype when E. coli was grown on blood agar. Furthermore, hemolytic activity was not detected in cell-free extracts of E. coli containing the αQ expression plasmid. Thus although αQ has a key role in HlyE function, it is unlikely that it has hemolytic activity per se.

The HlyE-L47S variant has a substitution located in the 310 ribbon and terminates. These two tyrosine residues are not sufficiently close to interact directly in the water-soluble form of HlyE, but their location suggests they have a key role in maintaining the integrity of the tail domain. The final two variants (N157H and Y165C) were located within the head domain of the protein and were notable because, although their in vitro activities were relatively high, the hemolytic zones surrounding colonies on blood agar were particularly small (Table II). This may indicate that these variants not only carry activity defects but are also compromised for export from E. coli.

Therefore, random mutagenesis has complemented and extended the previously reported site-directed mutagenic studies (5) by confirming the key role of αQ in HlyE function and identifying new regions of the protein that contribute to HlyE activity.

The Role of HlyE Cysteine Residues—The random mutagenic screen reported above indicates a key role for αQ in HlyE activity. This helix contains one (Cys-285) of the two cysteine residues of HlyE. The two cysteine residues (Cys-87 and Cys-285) lie opposite each other in the three-dimensional structure but do not form a disulfide bond in the native crystal structure.
TABLE II
Hemolytic activity of 10 HlyE variants

| HlyE variant | Codon change | Hemolytic activity (ng per μg of soluble cell protein) | Hemolytic activity |
|--------------|--------------|---------------------------------------------------|-------------------|
| HlyE         |              | 2.3                                               | 71.5 ± 7.5        |
| Y30C         | TAT-GTT      | 2.6                                               | 16.1 ± 3.5        |
| L47S         | TTA-TCA      | 2.5                                               | 26.1 ± 3.7        |
| Y76C (2)     | TAT-GTT      | 1.2                                               | 4.5 ± 0.8         |
| Y97H         | CAT-CAT      | 1.8                                               | 0.0               |
| N157H        | AAT-CAT      | 2.2                                               | 3.1 ± 0.0         |
| Y165C        | TAT-GTT      | 2.2                                               | 3.1 ± 0.0         |
| G180V, K275R | GGT-GTT, AAA-AGA | 2.2 | 16.1 ± 3.5 |
| R251K        | AGA-AAA      | 1.6                                               | 1.8 ± 0.8         |
| Y263H        | TAC-CAC      | 1.6                                               | 28.2 ± 0.0        |
| K290E        | AAA-GAA      | 2.3                                               | 19.6 ± 0.0        |

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Fig. 3. A disulfide form of HlyE. a, detection of an oxidized form of HlyE in medium of DH5omp GS1148 cultures. Western blot of non-reducing SDS-PAGE analysis of extracellular (lane 1) and intracellular (lane 2) HlyE. The positions of the reduced (SH) and oxidized (–S–S–) forms of HlyE are indicated. b, an intramolecular disulfide is introduced into HlyE in the E. coli periplasm. Non-reducing Western blot of cytoplasmic (lanes 1 and 2) and periplasmic (lanes 3 and 4) extracts of DH5omp GS1148 (lanes 1 and 3) and DH5omp GS1270 HlyE-C87S (lanes 2 and 4). The positions of the reduced (SH) and oxidized (–S–S–) forms of HlyE are indicated. c, the HlyE intramolecular disulfide observed in the x-ray structure of CuCl2-treated HlyE. Electron density maps of native HlyE (upper panel) show reduced cysteine residues and a Hg(II) (bold density) binding site; disulfide form of HlyE (lower panel) was obtained by oxidation with CuCl2. The upper map is a 2.0 Å (2Fα — Fβ) exp (αcalc (contoured at 1.5σ), the density for Hg is a (Fα — Fβ) exp (αcalc (contoured at 25σ). The lower map is a 2.8 Å (2Fα — Fβ) exp (αcalc map (contoured at 0.5σ).

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Substitutions in HlyE leading to reduced in vivo hemolytic activity were identified. The numbers in parentheses indicate independent isolates of a particular variant. The amount of soluble HlyE variant in test cultures was estimated by Western blotting with a polyclonal anti-HlyE serum. The blots were standardized with purified HlyE (10 ng) and analyzed by quantitative densitometry. Hemolytic activities of colonies expressing the indicated HlyE variants were measured on blood agar plates by calculating the area (mm2) of the zone of hemolysis surrounding the colonies. Four measurements were made for each variant, and the values are the averages with standard deviations. The in vitro hemolytic activities were used to determine the extent of the defect in each variant. Hemolytic activity of bacterial extracts was estimated after adjusting the volume of test extract so that equivalent amounts of HlyE were used. All assays were done in duplicate from three independent cultures, and the values are averages with standard deviations.

or in the E. coli cytoplasm (Table I), and neither is essential for activity (1). However, cysteine residues are rarely found in extracellular proteins unless they have some other role. Metal ion analysis by ICPMS eliminated the possibility of metal binding (Table I), but the proximity of the cysteine residues is very striking, and it seemed possible that the formation of an intramolecular disulfide, linking Cys-87 to Cys-285, could have some significance given the importance of α, the metal ion. Therefore, evidence for an oxidized form of HlyE was sought. Initial immunoblotting of N-ethylmaleimide-treated medium from cultures of DH5omp GS1148 indicated that up to 60% of exported HlyE contains an intramolecular disulfide as judged by the presence of a higher mobility, cross-reacting species, under non-reducing electrophoretic conditions (Fig. 3a). Western blots of periplasmic and cytoplasmic fractions from DH5omp GS1148 (wild-type HlyE) and DH5omp GS1270 (HlyE-C87S) revealed that HlyE is fully reduced in the E. coli cytoplasm but that an intramolecular disulfide is present in periplasmic HlyE (Fig. 3b). Densitometric analysis suggests that 60–70% of the total HlyE has been exported to the periplasm. Prolonged exposure of the isolated dithiol form of HlyE to oxygen did not result in disulfide bond incorporation. However, soaking crystals of HlyE in CuCl2, a compound known to assist in the formation of disulfides in proteins, was successful in generating the disulfide form. The structure reveals that the necessary rotations about the Cys-87 and Cys-285 C–C bonds do indeed occur, allowing the formation of a typical disulfide bond (S–S distance 2.03 Å) (Fig. 3c). Furthermore, comparing the crystal structures of the dithiol and disulfide forms of HlyE indicated that no other significant conformational changes had occurred. The addition of CuCl2 to HlyE in solution was accompanied by the loss of HlyE activity and reactive thiols (Table III). Activity was restored by the addition of reducing agent (dithiothreitol), and two HlyE variants with single cysteine replacements (HlyE-C87S, HlyE-C285S) were unaffected by Cu(II), indicating that it is only the disulfide bond that contributes to HlyE inhibition (Table III).

The Oligomeric State of HlyE in Solution and in Target Membranes—Previous gel filtration analyses using low initial concentrations (−0.05 mg ml−1) of HlyE indicated that HlyE activity was associated with monomeric protein (1). However, careful analysis of the elution profile of unaltered HlyE at higher protein concentrations (1.1 mg ml−1) revealed that although it is almost completely monomeric, there is evidence for a minor peak (∼5% of A280) equivalent to a HlyE 8–10-mer. Furthermore, incubation (37 °C for 2 h) of HlyE while maintaining its reduced state with dithiothreitol (2 mM) promoted the formation of HlyE oligomers (monomers, dimers, and 8–10-mers), the presence of HlyE in the equivalent fractions from the gel filtration column being confirmed by Western blotting. However, HlyE activity was only associated with the monomeric and dimeric forms and not with the higher aggregates. Therefore, the HlyE dimer observed in solution could be the same as that observed crystallographically (1). It is clearly capable of dissociation to allow red blood cell lysis and may be formed to bury the hydrophobic regions of HlyE in order to improve solubility of the exported protein. Linking α to the main body of the protein (by treatment with Hg(II), see below) tended to preserve the monomeric state, whereas single cysteine-substituted HlyE variants tended to aggregate, eluting mostly in the 8–10-mer range (Fig. 4a). This suggests that both
cysteine residues contribute to preventing HlyE aggregation in solution.

In solving the structure of HlyE, the Hg(II) derivative compounds ethyl mercury phosphate and 4-(chloromercuri)benzene sulfonic acid (PCMBS) have been used, and a single Hg(II) atom was found to cross-link the two cysteine residues, joining \( \alpha_G \) to the main four-helix bundle of the protein (1) (Fig. 3c). Consistent with the data obtained with Cu(II)-treated protein, HlyE activity was abolished by both Hg(II) salts, but HlyE-C87S was only partially inhibited, and HlyE-C285S was unaffected (Table III). Immunoblotting of membranes from red blood cells that had been exposed to inactive, Hg(II)-treated, HlyE revealed that linking the cysteines does not prevent binding to target membranes (Fig. 4b). It would therefore appear that restricting the mobility of \( \alpha_G \) by binding it to the rest of the protein, whether by direct disulfide bond formation or by Hg(II) cross-linking, abolishes HlyE activity by inhibiting pore formation at some stage after membrane association.

The observation that the inactive Hg(II)-treated HlyE protein still associated with target membranes but failed to oligomerize in solution prompted the use of the chemical cross-linking agent, dimethyl suberimidate, to determine the oligomeric status of membrane-associated HlyE. These studies confirmed that both Hg(II)-treated and untreated HlyE can interact with target membranes. However, in contrast to the HlyE in free solution, cross-linked species with relative molecular masses of 76,000 (2.2-mer), 180,000 (5.6-mer), and ~230,000 (7.2-mer), as well as monomers (<47,000), were observed with both untreated and Hg(II)-treated HlyE (Fig. 4c).

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On some blots additional cross-linked species with relative molecular masses of 90,000 (2.8-mer), 110,000 (3.5-mer), and 125,000 (3.9-mer) were observed (not shown). These sizes are consistent with HlyE dimers, trimers, tetramers, and octamers when it is realized that cross-linked molecules will also affect mobility. Similar experiments with samples not exposed to dimethyl suberimidate and separated on non-reducing SDS-PAGE before detection by Western blotting indicated that HlyE subunits associated with target membranes were not covalently linked. Thus combined with the gel filtration data, these observations suggest that the HlyE pore consists of a non-covalently linked assembly of eight HlyE monomers and that immobilizing \( \alpha_G \) inhibits activity at some point after membrane association and oligomerization. However, the possibility that the HlyE octamer represents the building block from...
which the mature pore is assembled cannot be discounted at this point.

**DISCUSSION**

The simple protocol for the isolation of HlyE described represents a significant improvement upon previous procedures. The high yields of pure protein have already been exploited in the structural analysis of HlyE and have now allowed some of the biochemical and biophysical properties of the protein to be determined.

The random mutagenic strategy adopted here to study structure-function relationships of HlyE has a major advantage over a directed approach because no pre-conceptions are involved. Previous studies have indicated that the β tongue is crucial for HlyE function (1, 5), but no activity-compromising substitutions were identified in the β tongue by using a random mutagenic screen, suggesting that single substitutions in this region are insufficient to generate a discernible phenotype. However, one activity-defective variant HlyE-G180V K275R contained a substitution in the loop between Gly-180 and Ala-183 and probably cause repositioning of the G180V replacement will disrupt the packing between Gly-180 and Ala-183 and probably cause repositioning of the β tongue itself, could affect its presentation to target membranes and receptor binding. The G180V replacement will disrupt the packing between Gly-180 and Ala-183 and probably cause repositioning of the β tongue. Previous findings that substitution K275A had little effect on HlyE activity (5) indicates that it is probably the G180V substitution that is so debilitating for this variant.

The activity-impairing substitution L47S located in the 3 10 bundle of the G180V replacement that is so debilitating for this variant.

The activity-impairing substitution L47S located in the 3 10 bundle of the tongue region (1), may indicate that the hydrophobic surface employed by HlyE to associate with target membranes extends beyond the β tongue itself.

The results of the random mutagenesis described here clearly highlight the crucial role of αG in HlyE activity (5 of the 10 activity-impaired variants had amino acid substitutions either within, adjacent to, or interacting with αG). Two activity-defective variants had substitutions in the loop between αG and αP suggesting that αG may hinge away from the main body of the protein during pore formation. A requirement for αG mobility is supported by the inhibition of HlyE activity upon disulfide bond formation or by Hg(II) cross-linking. Moreover, the reactivity of the two cysteine thiols, which are buried in the three-dimensional structure, suggests that αG can dissociate from the main four-helix bundle in solution to expose both cysteine residues. This mobility does not appear to be required for HlyE to associate or for initial oligomerization within target membranes, because the Hg(II)-treated protein still bound to red blood cells and formed octameric assemblies. Thus, the possibility that the tail domain (a 5-helix bundle including αG) of HlyE could be involved in penetrating target membranes via transition through a molten globule state remains remote (1).

It therefore seems that the step inhibited by Hg(II) must occur at a late stage of HlyE pore formation, perhaps in stabilizing the HlyE octamer. Dissociation of αG would expose a hydrophobic region of the four-helix bundle that may mediate subunit interactions that stabilize the pore in the membrane. However, such interactions would need to be avoided in the absence of a target, because oligomerization leads to inactivation. Therefore, an intramolecular disulfide introduced in the periplasm fixes αG to the main body of the protein, preventing premature oligomerization and allowing activation in reducing (anaerobic) environments, in accord with the anaerobic activation of hlyE expression (2). Alternatively, αG may be involved in opening the pore and may contribute to the central density observed in some previous EM images (1). However, if αG somehow plugs the pore, it would have to be within the channel rather than on the outside of the assembly.

A wide range of oligomeric states are found among bacterial pore-forming toxins. The pneumolysin pore consists of 30–50 non-covalently linked monomers (13), whereas the δ-toxin (14) and α-hemolysin (15) proteins of S. aureus are hexameric, the aerolysin from Streptococcus pneumoniae (16) is heptameric, and the V. cholerae cytolsin forms a pentameric pore (17). The gel filtration and cross-linking data presented here indicate that HlyE forms non-covalently linked octameric pores in target membranes and that, like pneumolysin, it can self-associate in solution (17). However, although gel filtration indicates that the soluble HlyE oligomers are mostly in the 8–10-mer range, it is not yet known if any component of the water-soluble aggregates resemble the membrane-associated pores. An assembly of eight HlyE monomers in the mature pore proposed here is consistent with the model based on structural information (1) and with the diameter of the HlyE pore estimated by osmotic protection assays (4).

In conclusion although it has been suggested that the C-terminal region (amino acids 282–303) that includes about half of αG is multifunctional, playing a role in secretion, membrane targeting, and pore formation (5), the data presented here suggest that it is not essential for HlyE export, membrane targeting, or initial oligomerization but rather that an immobilized αG inhibits oligomerization (and consequent inactivation) of HlyE in the absence of a target membrane and that αG mobility may be required to stabilize or open the HlyE pore.

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