Structure and Functional Characterization of Vibrio parahaemolyticus Thermostable Direct Hemolysin

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Thermostable direct hemolysin (TDH) is a major virulence factor of Vibrio parahaemolyticus that causes pandemic foodborne enterocolitis mediated by seafood. TDH exists as a tetramer in solution, and it possesses extreme hemolytic activity. Here, we present the crystal structure of the TDH tetramer at 1.5 Å resolution. The TDH tetramer forms a central pore with dimensions of 23 Å in diameter and ~50 Å in depth. π-Cation interactions between protomers comprising the tetramer were indispensable for hemolytic activity of TDH. The N-terminal region was intrinsically disordered outside of the pore. Molecular dynamics simulations suggested that water molecules permeate freely through the central and side channel pores. Electron micrographs showed that tetrameric TDH attached to liposomes, and some of the tetramer associated with liposome via one protomer. These findings imply a novel membrane attachment mechanism by a soluble tetrameric pore-forming toxin.

Vibrio parahaemolyticus is a Gram-negative marine bacterium known to be one of the major causes of pandemic seafood-borne gastroenteritis. V. parahaemolyticus possesses two circular replicons of 3.2 and 1.9 megabase pairs, which might confer an advantage by enabling DNA replication in seawater of lower temperature and/or low nutritional value (1, 2). Such an advantage would potentially increase risks of food intoxication by allowing explosive expansion of the population of the microorganism. Hemolysis on Wagatsuma agar (a blood agar), known as the Kanagawa phenomenon, is associated with human pathogenic strains of V. parahaemolyticus. A major virulence factor of this pathogen is the thermostable direct hemolysin (TDH)2 (3–5), which has a variety of biological activities including hemolytic activity, cardiotoxicity, and enterotoxicity. There are two copies of the tdh gene (or its homologue thrh) in pathogenic V. parahaemolyticus, indicating the importance of this exotoxin for survival of the organism (2). The mature form of TDH consists of 165 amino acids, including a single intramolecular disulfide bond, but no close homologue of TDH has been found in other organisms. The significance of Arg46, Gly62, Trp69, and Gly89 residues on hemolysis was determined by site-directed mutagenesis (3, 6).

The common features of the bacterial pore-forming toxin are as follows. 1) It is released as a soluble monomer into the extra-bacterial space. 2) It oligomerizes to form a pore at the host cell membrane (7, 8). An earlier study reported that TDH acted as a pore-forming toxin, creating a functional pore ~20 Å in diameter (reviewed in Ref. 3). We previously constructed a low resolution Cs symmetric model of tetrameric TDH in solution based on small angle x-ray scattering (SAXS), transmission electron microscopy (TEM), and analytical ultracentrifugation (9). However, the precise structure and the mechanism for its pore-forming toxicity are still unknown. Several bacterial toxins, including TDH, show paradoxical responses to heat treatment, known as the Arrhenius effect (10, 11). The abbreviations used are: TDH, thermostable direct hemolysin; SAXS, small angle x-ray scattering; CAPS, 3-(cyclohexylamino)propanesulfonic acid; TEM, transmission electron microscopy; SEC, size exclusion chromatography; HSQC, heteronuclear single quantum correlation; MD, molecular dynamic.
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11. The hemolytic activity of crude TDH is inactivated by heating to 60 °C but is reactivated by further heating for 15 min at 90 °C (11). The Arrhenius effect has been recognized for 100 years, and its underlying molecular mechanism was finally unraveled in our previous report (12). TDH exhibits a thermore- duent “reversible amyloidogenic property” according to circular dichroism spectra, electron micrographs, and a thiofla- vin T assay. TDH exhibited an ion channel-like activity in lipid bilayers with relatively low ion selectivity (13). Here, we present the first tetrameric structure of a pore-forming toxin, a simulation of its channel activity at atomic resolution, and the mechan- ism of lipid membrane association by tetrameric TDH.

EXPERIMENTAL PROCEDURES

Structure Determination—Crystals were grown in 100 mM CAPS (pH 10.6) containing 1.0 mM lithium sulfate in hanging drops. Diffraction data were collected at SPring-8 and the Photon Factory. The structure was determined by single wave- length anomalous diffraction using a gold derivative. The gold derivative crystals were prepared by soaking in the crystalliza- tion buffer including 37 mM KAu(CN)2. All diffraction data were indexed, integrated, scaled, and averaged using the pro- gram HKL2000 (14). The intensities were processed to structure factors using the CCP4 suite of programs. The structure was determined by single anomalous diffraction by a gold deriv- ative using the programs SOLVE (15) and RESOLVE (16, 17). The initial model was built by the program RESOLVE, and the model was automatically rebuilt using the program ARP/ wARP. The model was improved manually using the program COOT (18) and was refined using the program CNS (19) and then refined using REFMAC (20) with an anisotropic B-factor. The geometry of the refined model was validated by the pro- gram PROCHECK (21). Data collection, phasing, and refine- ment statistics are summarized in supplemental Table 1.

Purification, Site-directed Mutagenesis, and Bioassay—Wild-type and mutant TDH were purified, and their hemolytic activities were assayed (12). Site-directed mutagenesis was performed on V. parahaemolyticus tdh in the pKK223-3 vector using the QuikChange site-directed mutagenesis kit (Stratagene) with over- lapping mismatching oligonucleotide primers. The PCR prod- ucts were transformed into Escherichia coli JM109 competent cells, and all mutations were verified by DNA sequencing. The oligonucleotide primers are shown in supplemental Table 2.

Single Particle Image Analysis and Liposome Preparation—Solutions containing 0.13 mg/ml TDH were diluted with 10 mM sodium phosphate at pH 7.0. For negative staining, 4 μl of sample solution was applied to a copper grid supporting a continu- ous thin carbon film, left for 1 min, and then stained with 3 drops of 2% uranyl acetate. The random conical tilt method (22) was used for initial three-dimensional model generation; thus, every specimen field was recorded twice, first tilted (45°) and then untitled. Images were recorded at nominal magnification of ×48,000 on Fuji FG film using a JEM100CX electron micro- scope (JEOL), operated at an accelerating voltage of 100 kV. The films were digitized by a CCD scanner (Nexscan 4100, Hei- delberg, Inc.) at a 10-μ pitch (corresponding to 2.1 Å at the specimen). EOS (23), SPIDER (24), IMAGIC (25), and EMAN (26) were used for image analysis. The initial three-dimensional map was calculated by SIRT (27) using 527 untitled images of the TDH tetramer. The final three-dimensional model was re- constructed from 2,516 images by projection matching.

Liposomes were prepared from egg yolk 1,2-sn-phosphatidyl- choline, cholesterol, and trisialoganglioside-G1b (molar mixing ratio of 65:20:15). Images were recorded using a BioScan CCD camera (Gatan) and a JEM1010 microscope with an actual pixel size of 3.1 Å/pixel.

Molecular Dynamic Simulations—Molecular dynamic (MD) simulations were carried out with the program MARBLE (28) using the CHARMM22/CMAP force field (29). The initial structure was placed in a periodic box with dimensions 102 Å × 102 Å × 102 Å containing ~30,000 TIP3P water molecules (30) and 36 counter ions. The crystal structure of the TDH tetramer was used as the initial structure, in which form A of multiple conformers was selected, and the missing N-terminal residues 1–11 were modeled as random coil structures. The initial sys- tem was subjected to 1,500 steps of energy minimization using the steepest descent method, followed by a 1-ns equilibration MD run under constant volume and temperature conditions at 300 K with positional harmonic restraints to the crystal structure. After the equilibration MD run, a 10-ns production run was performed under constant pressure and temperature con- ditions at 300 K and 1 atm. Long range electrostatic interactions were calculated using the particle mesh Ewald method (31). The symplectic integrator for rigid bodies was used with a time step of 2 fs, in which water and CH3NH2 (x = 1, 2, 3), SH, and OH groups were treated as rigid bodies (28).

To analyze the TDH tetramer pore, the pore axis calculated from each snapshot of the MD trajectory was aligned to the z axis, and the origin (z = 0) was set to the center of mass of the main chains of Val19 and Val149. To identify the volume occup- ied by water inside the pore during the MD simulation, the pore in the range of −21 Å ≤ z ≤ 21 Å was divided into disk- shaped segments centered on the pore axis. The thickness of the disk segments was set to 3 Å. For each disk segment, the radial density profiles of water were calculated using a 1 Å bin size for the radii. The volume occupied by water inside the pore was defined as the region in which the water density was >20% that of bulk water. Molecular graphic images were produced by using PyMOL (32) and VMD (33).

Sedimentation Velocity and Size Exclusion Analysis—Sedi- mentation velocity experiments were performed using an Op- tima XL-I analytical ultracentrifuge (Beckman). TDH (1.0 mg/ml) was dissolved in 20 mM sodium phosphate (pH 7.4), 100 mM NaCl, and ultracentrifuged at 40,000 rpm (wild-type TDH) or 50,000 rpm (R46E) at 20 °C (5-min intervals). The data were analyzed by the van Holde and Weischet method with UltraScan. For size exclusion analysis, TDH was applied to a Superose 6HR column using an AKTA purifier (Amersham Biosciences-Pharmacia).

Small Angle X-ray Scattering—The beads model of TDH was reconstructed based on an earlier SAXS profile of TDH (8). Twenty SAXS models were calculated using the program package Gloopy in CREDO (Svergun et al., 34), and an averaged most populated volume envelope was calculated using the DAMAVER suite (34). The resulting structure was docked into a crystal structure using Situs version 2.3 (35).
RESULTS

TDH Tetramer with a Central Pore—The crystal structure of TDH was determined at 1.50 Å resolution (supplemental Table 1). There was a single TDH protomer per asymmetric unit. TDH adopted a β-sandwich structure composed of 10 β-strands flanked by two helices, α1 and 3₁₀ (Fig. 1A and supplemental Fig. 1A). The protein has one intramolecular disulfide bond between Cys¹⁵¹ in β₁₀ and Cys¹⁶¹ in the 3₁₀ helix. These cysteines are also conserved in TDH-related hemolysin (supplemental Fig. 1B). There was no electron density observed for the N-terminal region (residues 1–11), suggesting that this region is highly flexible or adopts more than one conformation. A search for homologous structures using the DALI server revealed that the TDH monomer structure was similar to those of equinatoxin II from sea anemone Actina equina (Z-score = 10.8) and Xerocomus chrysenteron, an ectomycorrhizal fungus lectin (Z-score = 8.8) (supplemental Fig. 1C), although TDH had no remarkable sequence similarity to either equinatoxin II or Xerocomus chrysenteron lectin. The asymmetric unit contained the TDH protomer, whereas TDH formed a tetramer with the monomers arranged around a crystallographic 4-fold axis. This type of soluble tetrameric assembly has not been observed in structures of other pore-forming toxins, including equinatoxin II. Remarkably, the tetramer had a central pore with a diameter of 23 Å and a depth of ~50 Å (Fig. 1, B–D). The tetramer superimposed well on the three-dimensional reconstruction particle generated by TEM (Fig. 1, C and D). Thus, the tetrameric structure was not an artifact of crystallization, as TDH formed the same stable structure in solution. It should also be noted that the three-dimensional TEM analysis did not produce a structure for the N-terminal region that was disordered in the crystal structure. Residues located in β3, β4, β9, and the C-terminal region were involved in tetramer formation (Fig. 1E). In particular, R₄₆ was involved in multiple interactions at the protomer-protomer interface. The guanidium group of Arg₄₆ formed an ion pair network with both the carboxyl group of Glu¹³₄ and the C-terminal carboxyl group of Gln¹⁶⁵ in the adjacent protomer. Furthermore, the guanidium group of Arg₄₆ formed a π-cation interaction with the aromatic ring of Tyr¹⁴⁰ in the adjacent protomer. Arg₄₆ and Tyr¹⁴⁰ are absolutely conserved in TDH and TDH-related hemolysin of

FIGURE 1. Monomeric and tetrameric structures of TDH. A, ribbon representation of the TDH monomer. A disulfide bond is shown by stick representation. B, Ca-tracing of the TDH tetramer. Individual protomers are shown as green, cyan, magenta, and yellow wire models. The molecular surface is shown in transparent representation. Water molecules are indicated by red spheres. The crystallographic 4-fold axis is shown by a black square. Top (C) and side (D) views of TDH tetramer superimposed to three-dimensional reconstruction from 2,516 electron microscopic images of the TDH tetramer. E, intersubunit interactions. Only two protomers are shown in green and cyan ribbon representation. Residues interactions with Arg₄₆ are only shown by stick models (left panel). A close-up stereo view (right panel) of intersubunit interactions. Ionic interactions and hydrogen bonds are shown by dashed lines.
other bacterial strains, indicating that the π-cation interaction plays a significant role in the structure and function of TDH.

Tetramer Form Indispensable for Hemolysis—The present study reveals that TDH forms a tetramer with a central pore, as described above. To examine correlations between the quaternary structure and hemolytic activity, several mutant proteins were generated with substitutions of residues involved in the π-cation interaction (R46E, Y140F, and Y140A). Analytical ultracentrifugation revealed that the R46E mutant existed as a monomer (Fig. 2, A and B). In contrast, the Y140F mutant existed as a tetramer (Fig. 2E). Because this substitution replaced the aromatic Tyr side chain with that of Phe, the π-cation interaction could be maintained. Based on size exclusion chromatography (SEC) analysis, the Y140A mutant existed predominantly as a monomer (14/15) with only a minor portion forming tetramers (1/15) (Fig. 2E). These findings strongly indicated that the π-cation interaction involving Arg46 and Tyr140 was crucial for tetramer formation. Hemolysis assays of wild-type and mutant proteins were also performed. Wild-type TDH exhibited 50% hemolytic activity at a toxin concentration of 0.20 μM. Interestingly, the R46E mutation abolished hemolytic activity. Although the Y140F mutant retained full activity (0.17 μM), the Y140A mutation significantly impaired the activity (0.63 μM) (Fig. 2F). The tetramer formation and hemolytic activity were highly correlated, strongly indicating that tetramer formation was indispensable for hemolytic activity and that the central pore formed by tetramer assembly functioned as a channel during hemolysis.

\[1H-15N\] NMR heteronuclear single quantum correlation (HSQC) spectra of the R46E mutant showed that the peaks appeared well separated, indicating formation of a stable protomer tertiary structure with extensive β-strands (supplemental Fig. 3A). However, the number of peaks observed was less than expected, and some peaks were considerably broadened. To identify these broadened or missing peaks, doubly [15N/13C]-labeled R46E were prepared, and a series of three-dimensional spectra were acquired. In supplemental Fig. 3B, the residues derived from the unambiguously assigned observable peaks were mapped onto the tertiary structure. Clearly, most of the unassigned residues were located in the interface region between the two adjacent protomers. These peaks were likely broadened owing to an exchange among multimeric states. The data indicated that R46E was in equilibrium highly skewed to the monomer state and that the tetramer population was under the detection limits of SEC or analytical ultracentrifugation (Fig. 2 and supplemental Fig. 2).
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Structure of the Flexible N Terminus—As mentioned above, the pore structure of the TDH tetramer is essential for hemolysis. However, the binding mode of TDH to lipid membranes has not been characterized. Therefore, the electrostatic potential of the tetramer surface was calculated, but it did not reveal any large hydrophobic patches (supplemental Fig. 4). The N-terminal Pro-rich region (FELPSVPFPAP), however, was apparently hydrophobic, but its structure was not discernable by x-ray crystallography, three-dimensional TEM (Fig. 1), or NMR studies (supplemental Fig. 3). Our interest was to reveal the spatial relationship between the unstructured N-terminal region and the pore structure. The ab initio model of TDH including the N-terminal region from SAXS data were relatively flattened, oblate, and widened compared with the crystal structure without the N terminus (9). This earlier SAXS model provided the diameter of tetrameric TDH (d_{max} of 100 Å), which was larger than that of the tetramer in the crystal structure (Fig. 1), indicating that the flexible N-terminal 11 residues may alongside the tetramer. In the present study, the N-terminal region was modeled by using the atomic coordinates of the tetramer without the N-terminal regions and the previous SAXS profile (Fig. 3). The averaged most populated volume envelope from the 20 SAXS models showed that the N-terminal region projected from the central pore and was found primarily on the longitudinal side of the tetramer. The structural model indicated that the missing N-terminal region was exposed to solvent and adopted a highly mobile conformation.

Molecular Dynamic Simulation of TDH—As described above, tetrameric TDH had a central pore. Water molecules existed in the pore, indicating that it could function as a channel to permit the flow of ions or waters. Molecular dynamic (MD) simulations were performed to evaluate this idea. The root mean square deviation of Cα atoms in secondary structures from their counterparts in the crystal structure were <1.0 Å during the 10-ns MD simulation, suggesting that the structure of the TDH tetramer with a large pore was stable. The region where the water density was >20% the density of bulk water during the MD simulation was defined as inside the pore. Fig. 4A shows the profile of the pore diameters along the axis. The averaged diameter of the pore was 23.0 ± 2.1 Å, and the narrowest diameter of the pore was 18.2 Å (z = −6 Å; the definition of z was described in the “Experimental Procedures”). The small fluctuations of the pore diameters suggested that the pore size was stable throughout the MD simulation. TDH forms a toxic pore on the erythrocyte membranes. This was visualized by low resolution TEM, which indicated the functional diameter of the toxic pore was ∼20 Å (reviewed in Ref. 3), in good agreement with the present results. Interestingly, in addition to the central pore, four adjacent pores located at z = −9 Å ≤ z ≤ −6 Å were found at the interface of two adjacent protomers (Fig. 4B). These side pores were also observed in the images of the single particle TEM experiments (Fig. 1). A further analysis was carried out of water permeation through the TDH pore during the MD simulation. The averaged number of water molecules inside the pore was 614.6 ± 13.6, and 695 full permeation events of water through the central pore were observed during the 10-ns MD simulation. The averaged passage time of the permeation events was 1.1 ± 0.7 ns. Fig. 4C and supplemental Movie 1 show an example of the water permeating through the central channel. Because this MD simulation was performed in aqueous solution with no osmotic pressure difference between the two entrances of the pore, no net water flux through the pore was observed. During the MD simulation, 309 permeation events of water molecules through the side channels were also detected, and the average passage time for water permeation through the side channels was 0.7 ± 0.7 ns. For calculating side channel permeation events, a pore boundary with a length of the radius plus 5 Å away from the pore axis was used. Fig. 4D and supplemental Movie 2 show examples of such permeation events in which a water molecule entered into the pore from a side channel and exited from the pore entrance. Those results indicated that both the central and side pores function as channels during hemolysis.

Diagonal Attachment of Tetrameric TDH—The crystal structure of tetrameric TDH showed it was hydrophilic in nature. The electrostatic potential of the tetrameric TDH showed that it had a small hydrophobic patch at the N-terminal external pore region (supplemental Fig. 4). To address the initial membrane association mechanism by TDH, the ganglioside G_{1-12} containing liposomes were incubated with or without TDH (Fig. 5, A and B) at 37 °C for 15 min. TEM analyses showed that tetrameric TDH attached to the liposome with one protomer (diagonal attachment, Fig. 5C), or with two protomers (Fig. 5D) in 15 min. These findings provided the evidence that TDH maintained its tetrameric structure during initial membrane association.

FIGURE 3. SAXS analysis of TDH. A, red circles indicate the raw data measured at pH 7.0, 20 °C (9). The theoretical profile calculated by 20 models is drawn as a black line (±S.D.). The protein concentration was 9.6 mg/ml. The superimposed volume envelopes of TDH constructed from twenty SAXS models. B, top view. C, side view. Molecular surface from the crystal (blue), the most populated model from SAXS analysis (red sphere, N terminus 11 residues; white sphere; amino acids 12–165), and the second to 20th most highly populated structures calculated for the N-terminal 11 residues (green).
DISCUSSION

The structure-based mutagenesis results showed the importance of the π-cation interaction between Arg46 and Tyr147 for stabilization of the tetrameric structure. Hemolytic activity assays of wild-type and mutant toxins showed that the maintenance of tetrameric structure with a central pore is indispensable for biological activity (Fig. 2A). Two-dimensional HSQC NMR spectroscopy (supplemental Fig. 3A) indicated the existence of a small portion of monomers in equilibrium with the tetramers for the mutant R46E, suggesting the interaction between E138 and Q165 also contributed to stabilization of the tetrameric structure. However, the N-terminal region (residues 1–11) was disordered in the crystal structure, and SAXS analysis revealed that this region was exposed to solvent and highly flexible.

To obtain more experimental evidence that supports water transport through tetrameric TDH, the particle size distribution of human red blood cells was measured by an electrozone-sensing particle size analyzer (CDA-1000, Sysmex), and the diffusional water permeability thorough them was measured by NMR (DRX-500, Bruker). The increased volume and the decreased diffusion constants of human red blood cells after treatment with wild-type TDH or a mutant, R46E, seemed to be consistent with the limited diffusion behavior of the trapped influx of water into the intracellular space (data not shown). However, we could not estimate the effect of hemolysis during the experiments on the observed diffusion constants or the precise amount of membrane-bound TDH, so we failed to calculate the hypothetical pore size by the pulsed field gradient NMR analyses.

Comparison of the TDH channel with a water channel protein, aquaporin, is useful for characterizing the TDH pore. The diameters of the water pores formed by aquaporin 1 (AQP1) are ~3–5 Å (36), which is slightly larger than the diameter of a water molecule (2.8 Å). The narrowness of the water pore in AQP1 is one of the major means by which it excludes ions and larger molecules, resulting in its selectivity for water molecule. The much larger size of the TDH pore indicates that it may exhibit low selectivity against other molecules or ions. In fact, experiments measuring ion channel-like activities of TDH showed low ion selectivity (13, 37). A previous MD simulation of α-hemolysin (38), which has a pore size comparable to that of TDH, indicated that ions could permeate through the pore. We also found additional side pores permitting the flow of water molecules in the protomer-protomer interface. In fact, it is known that most of the pore-forming toxins display unitary channel conductance. In contrast, the TDH channel showed low ion selectivity and three types of conductance (13). The observed variability in conductance might result from the additional pores in TDH. Alternatively, this electrophysiological property might indicate that TDH adopts several oligomeric states and/or undergoes conformational changes during hemolysis at the membrane. Similar side channels were found in the extramembranous region of α-hemolysin, and water molecules permeating through the side channels were also observed in an MD simulation of α-hemolysin (38). The mutagenesis experiments of TDH showed that C151S and C161S mutant proteins had increased hemolytic activity (6). These results suggested that the side channels might influence the hemolytic activity of TDH, because Cys151 and Cys161 were adjacent to the side channel as shown in Fig. 4B.

The amino acid residues reported to be responsible for its hemolytic activities were mapped (i.e. Gly62, Trp65, Thr67, and Gly69) (3, 6) (supplemental Fig. 5). All of these residues are located in its N-terminal face, with Trp65 and Thr67 in the vicin-
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ity of the α1 helix. Both the W65L and T67I mutants bound to the lipid membrane but lost their hemolytic activity (supplemental Fig. 6).

The TDH tetramer showed a characteristic attachment with liposomes after 15 min of incubation by TEM analyses (Fig. 5). In conjunction with the previous report of the pore formation

FIGURE 5. Attachment of TDH to lipid membrane. TEM analysis of 1 mM liposomes incubated with 0.019 mg/ml TDH (A) or without TDH (B) at 37 °C for 15 min. Black bar = 100 nm. Representative electron microscopic images of TDH and liposome. TDH attached to the liposome with one corner (C), and with two corners (D). Red bar = 10 nm. TDH was traced in red, and liposome was in blue (lower panel).
by TDH (reviewed in Ref. 3), this unprecedented membrane association will shed light upon the molecular mechanisms of the small oligomerized toxic proteins.

The results of the present study indicate that the tetramer formation of TDH is responsible for membrane disruption, and molecular dynamic simulations show the central and side pore channels also participate in this disruption. Future studies will address: 1) the precise role of the flexible N-terminal region, 2) the determination of the region involved in the diagonal attachment, which might also trigger the tetramer insertion at the membrane, and 3) the evidence of water diffusion, provided by analyzing a series of mutant toxin crystals.

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