The β-Prism Lectin Domain of Vibrio cholerae Hemolysin Promotes Self-assembly of the β-Pore-forming Toxin by a Carbohydrate-independent Mechanism*

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Background: Vibrio cholerae hemolysin is a pore-forming toxin with a β-prism lectin domain. Because deletion or point mutation of the lectin domain seriously compromises hemolytic activity, it is thought that carbohydrate-dependent interactions play a critical role in membrane targeting of VCC. To delineate the contributions of the cytolsin and lectin domains in pore formation, we used wild-type VCC, 50-kDa VCC (VCC50) without the lectin domain, and mutant VCCD617A with no carbohydrate-binding activity. VCC and its two variants with no carbohydrate-binding activity moved to the erythrocyte stroma with apparent association constants on the order of 107 M−1. VCC, which induces lysis of mammalian cells by forming transmembrane 14-stranded β-barrel channels 0.9 nm in diameter in the plasma membrane bilayer (3, 5), triggers vacuolation (6) and apoptosis (7, 8) of epithelial and immune cells at sublytic concentrations. VCC, which is enterotoxic in rabbit ligated ileal loop (9) and lethal in mouse pulmonary models (10), is widely thought to enhance virulence of V. cholerae, the organism responsible for human cholera (11).

The hallmark of the mechanism of membrane channel formation by β-PFTs is the switch-over of the water-soluble cytolsin monomer to an amphipathic oligomer with a transmembrane β-barrel at the surface of the target cell (12–15). The initial event in this transformation is concentration of the toxin on the target cell surface. The PFTs exhibit specificities for a wide range of cell surface molecules, e.g. cholesterol (16), glycosylphosphatidylinositol-anchored glycoproteins (17), and the human complement receptor CD59 (18). Although hemolysis of rabbit erythrocytes by VCC is competitively inhibited by asialofetuin and glycoproteins with multiple β1-galactosyl residues (19), the evidence for cell surface carbohydrates acting as functional receptors for VCC is not convincing for several reasons. First, VCC shows strong preference for cholesterol- and sphingolipid-rich vesicles (2), presumably through amphipathicity-driven interactions (20). Second, preincubation of VCC with asialofetuin inhibits its interaction with phosphatidylcholine/cholesterol (PC/CL) liposomes, a process that has nothing to do with interaction of the toxin with carbohydrate receptors (19). Third, preincubation of rabbit erythrocytes with galactose-specific lectins makes the cells more susceptible to lysis by VCC (19). The interest in the carbohydrate-binding activity of VCC revived with the identification of two contiguous lectin-like folds at the C terminus in the three-dimensional structure of the toxin (21). Deletion of the 15-kDa β-prism fold bearing sequence and structural homology to the carbohydrate-binding domain during toxin assembly generates entropy to push oligomerization and thereby promote pore formation.

Results: Although amphipathicity-driven interactions with lipids dominate membrane targeting, relocation of the lectin domain during toxin assembly generates entropy to push oligomerization and thereby promote pore formation.

Conclusion: The lectin domain promotes toxin assembly by a carbohydrate-independent mechanism.

Significance: A novel role for the lectin domain in protein oligomerization is revealed.

Vibrio cholerae cytolsin/hemolysin (VCC) is an amphipathic 65-kDa β-pore-forming toxin with a C-terminal β-prism lectin domain. Because deletion or point mutation of the lectin domain seriously compromises hemolytic activity, it is thought that carbohydrate-dependent interactions play a critical role in membrane targeting of VCC. To delineate the contributions of the cytolsin and lectin domains in pore formation, we used wild-type VCC, 50-kDa VCC (VCC50) without the lectin domain, and mutant VCCD617A with no carbohydrate-binding activity. VCC and its two variants with no carbohydrate-binding activity moved to the erythrocyte stroma with apparent association constants on the order of 107 M−1. However, loss of the lectin domain severely reduced the efficiency of self-association of the VCC monomer with the β-barrel heptamer in the synthetic lipid bilayer from ~83 to 27%. Notably, inactivation of the carbohydrate-binding activity by the D617A mutation marginally reduced oligomerization to ~77%. Oligomerization of VCC50 was temperature-insensitive; by contrast, VCC self-assembly increased with increasing temperature, suggesting that the process is driven by entropy and opposed by enthalpy. Asialofetuin, the water-soluble, less amphipathic oligomer variant with reduced ability to penetrate the bilayer.

Vibrio cholerae cytolsin/hemolysin (VCC)3 is a water-soluble β-pore-forming toxin (β-PFT) (1–3) with a native molecular mass of 65 kDa (4). VCC, which induces lysis of mammalian cells by forming transmembrane 14-stranded β-barrel channels 0.9 nm in diameter in the plasma membrane bilayer (3, 5), triggers vacuolation (6) and apoptosis (7, 8) of epithelial and immune cells at sublytic concentrations. VCC, which is enterotoxic in rabbit ligated ileal loop (9) and lethal in mouse pulmonary models (10), is widely thought to enhance virulence of V. cholerae, the organism responsible for human cholera (11).

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domain of the plant lectin jacalin reduces hemolytic activity by ~1000-fold (22) and profoundly influences apoptotic and immunoregulatory activities (23). Interestingly, the role of the β-prism lectin domain in asialofetuin-binding and pore-forming activities critically depends on a single Asp<sup>117</sup> residue (24, 25).

To define the role of the β-prism lectin domain of VCC in hemolysis, we first considered how indispensable the role of carbohydrate-dependent interaction of VCC in binding to the rabbit erythrocyte stroma is. Recent structural investigations show significant rearrangement of the lectin domain during assembly of the membrane-inserted form of the VCC heptamer (26). We show that movement of the β-prism lectin domain during toxin assembly produces substantial entropy that should drive toxin assembly to completion. This is apparently a novel role of the lectin domain unrelated to its interaction with carbohydrate ligands. Finally, we show that asialofetuin induces oligomerization of the VCC monomer to a species that has a similar stoichiometry and morphology as the membrane-inserted oligomer but is more polar and less competent to insert into the bilayer.

**EXPERIMENTAL PROCEDURES**

**Protein Purification and Assay of Hemolytic Activity**—Mature 65-kDa VCC and its 50-kDa variant (VCC<sup>50</sup>) lacking the β-prism lectin domain were prepared from a recombinant source. Briefly, pro-VCC, which includes the 14-kDa prodomain (amino acid residues 26–741 of 82-kDa prepro-VCC) (27), was cloned into the pET32a(+) expression vector (Novagen) and transformed into SHuffle™ T7 competent *Escherichia coli* cells (New England Biolabs), which provided a nonreducing cytosol appropriate for proper folding of the toxin. To prepare VCC<sup>50</sup>, a stop codon was introduced after the cytolytic domain, and to obtain the mutant VCC<sup>D617A</sup>, a point mutation was introduced into the pro-VCC nucleotide sequence (24, 25). The proteins were purified to homogeneity by hydrophobic interaction chromatography on phenyl-Sepharose CL-4B, followed by elution with ethylene glycol (22). Protein concentrations were determined by using extinction coefficients of 1.60, 1.60, and 1.29 for 1 mg/ml solutions of VCC, VCC<sup>D617A</sup>, and VCC<sup>50</sup>, respectively.

The VCC heptamer formed in PC/CL liposomes was solubilized in 1% octyl glucoside in 50 mM Tris-HCl (pH 8.0) and separated from unconverted monomer by Sepharose CL-4B, followed by elution with ethylene glycol (22). Protein concentrations were determined by using extinction coefficients of 1.60, 1.60, and 1.29 for 1 mg/ml solutions of VCC, VCC<sup>D617A</sup>, and VCC<sup>50</sup>, respectively.

The VCC heptamer formed in PC/CL liposomes was solubilized in 1% octyl glucoside in 50 mM Tris-HCl (pH 8.0) and separated from unconverted monomer by Sepharose CL-4B chromatography (GE Healthcare). The oligomer induced in water in the presence of asialofetuin was purified by chromatography on DEAE-Sepharose (GE Healthcare).

The specific hemolytic activity was defined as the lowest toxin concentration causing 50% hemolysis of a 1% suspension of erythrocytes in PBS in 60 min at 25 °C. The kinetics of hemolysis was assayed by monitoring the decrease in light scattering by a suspension of erythrocytes at 600 nm (19).

**Triton X-114 Partitioning**—To study protein amphipathicity, proteins were dissolved in 2% Triton X-114 (Sigma) at 4 °C, and the solution was separated into two phases by raising the temperature to 25 °C (28). Following centrifugation at 10,000 × g for 10 min at 25 °C, equal volumes of aliquots were withdrawn from the water- and detergent-rich phases. Proteins were precipitated at 4 °C with 9 volumes of acetone and examined by SDS-PAGE (29).

*Asialofetuin*—Asialofetuin was prepared from fetuin (Sigma) by treatment with neuraminidase in 50 mM sodium acetate (pH 5.0) containing 1 mM CaCl<sub>2</sub> (30). The N-linked oligosaccharide was removed by overnight treatment with peptide N-glycosidase F (Sigma) in 250 mM sodium phosphate buffer (pH 7.8) containing 2.5% Triton X-100 at 37 °C (31). The O-linked oligosaccharide units of asialofetuin were stripped off by reductive cleavage with sodium borohydride under mild alkaline conditions (32).

**Liposome Preparation**—The PC/CL (1:1 by weight) liposome was prepared as described previously (20). The PC/CL film was dispersed in PBS and sonicated for conversion of multilamellar vesicles to unilamellar ones. The size uniformity was ensured by gel filtration on Sepharose CL-4B.

**Binding Studies**—VCC binds to membrane vesicles by two distinct modes, viz., carbohydrate-dependent interaction and amphipathicity-driven movement of the toxin from water to the vesicle, exemplified by binding of the toxin to asialofetuin (19) and PC/CL liposomes (2, 20), respectively. We used ELISA to quantify interaction of VCC and its variants with asialofetuin and rabbit erythrocyte stroma as described previously (19). Because membrane targeting of the toxin might be affected by the way vesicles coat the microtiter wells, for instance as a monolayer or a bilayer, and also by the use of detergent during washing, we monitored interaction of the toxin with the erythrocyte stroma by a variation of the ELISA technique described below. In both of these methods, we used the stroma prepared from rabbit erythrocytes by hypotonic shock with 5 mM sodium phosphate (pH 8.0).

The method employed for quantifying interaction of VCC and its variants with the erythrocyte stroma differs from the conventional ELISA technique basically in two respects: it avoids the presence of the detergent Tween 20 in the wash buffer and eliminates the immobilization of the stroma on polystyrene microtiter plates. The stroma (100 μl containing 300 μg of protein) was incubated in triplicate with various concentrations of the toxin in 1.5-ml microcentrifuge tubes with gentle shaking at 25 °C. The stroma was pelleted by centrifugation at 20,000 × g for 10 min and washed three times with PBS. The stroma was incubated with defatted milk powder (0.4% in PBS; Bio-Rad) for 1 h and washed as before. The pellet was suspended in PBS and incubated with rabbit anti-VCC antiserum (1:500) for 1 h at 25 °C. Following centrifugation, washing with PBS, the dispersed pellet was incubated with anti-rabbit IgG conjugated with horseradish peroxidase (1:1000; Sigma) for 30 min at 25 °C. The suspension was centrifuged, washed with PBS, and incubated with 1 mg/ml o-phenylenediamine in 50 mM sodium citrate (pH 4.5) containing 1 μl/ml H<sub>2</sub>O<sub>2</sub>. Color was allowed to develop until the blank without the toxin showed signs of producing color. The mixture was centrifuged at 20,000 × g for 5 min, and the supernatant was made 1 N with sulfuric acid. The toxin bound to the stroma was quantified by monitoring the absorbance at 490 nm.

**Self-assembly in Water and Effect of Temperature and Water-soluble Glycoproteins**—VCC kept in water for a sufficient length of time consists of a mixture of the monomer and the
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SDS-stable hemolytically inactive oligomer (33). Because there was no perceptible change in the relative amounts of the monomer and oligomer when the system was kept at 10 °C for 7 days in comparison with what was observed after an incubation time of only 6 h at the same temperature, we presumed that the system could reach a steady state in 6 h. In this work, we used an incubation time of 16 h to ensure attainment of equilibrium. In such a system, the monomer and oligomer exist according to the following equilibrium stoichiometry: 7 VCC ⇌ VCC7. The equilibrium constant \( K_e \) for self-assembly of the cytolysin in water is given by \( K_e = [\text{VCC}_7]/[\text{VCC}]^7 \). The cytolysin monomer and oligomer were separated by SDS-PAGE on 7.5% gel and stained with Coomassie Brilliant Blue, and the band intensity was quantified in a densitometer. The molar concentrations of the monomer and oligomer were calculated from knowledge of the relative intensities of the two components and the total cytolysin concentration in the sample loaded on the gel. The heptameric stoichiometry of the oligomer was taken into account while calculating the molarity of the oligomer.

For calculation of enthalpy of self-assembly, the cytolysin was kept in PBS at 10, 15, 20, 25, and 30 °C for 16 h, and the equilibrium constants for self-assembly were determined as described above. The enthalpy of self-assembly \( (\Delta H^o) \) was calculated by using the van’t Hoff equation \( (d \ln K/d(1/T) = -\Delta H^o/R) \) from the slope of the plot of \( \ln K \) versus \( 1/T \).

Transmission Electron Microscopy—VCC oligomers generated with PC/CL liposomes and in asialofetuin were negatively stained with 2% uranyl acetate and examined using a FEI Tecnai 12 BioTwin transmission electron microscope.

RESULTS AND DISCUSSION

C-terminal β-Prism Lectin Fold and Binding to Asialofetuin—Wild-type VCC, mutant VCCD617A, and the 50-kDa variant without the β-prism lectin fold at the C terminus (VCC50) caused 50% hemolysis of a 1% suspension of rabbit erythrocytes at 25 °C at 50, 800, and 40,000 pM, respectively, corresponding to relative specific hemolytic activities of 800, 50, and 1. Binding of VCC to asialofetuin was not affected by enzymatic removal of N-linked oligosaccharide moieties; rather, the binding was abolished by hydrolysis of the O-linked disaccharide Galβ1–3GalNAc by alkaline borohydride treatment (Fig. 1), indicating that O-linked rather than N-linked sugars of asialofetuin are recognized by VCC. VCCD617A and VCC50 did not show any affinity for the glycoprotein (data not shown). Notably, truncation of the domain caused a more drastic decrease in hemolytic activity in comparison with the effect of inactivation of lectin-like activity produced by substitution of Asp617 with Ala. Our data are similar to those reported by Levan et al. (25), but different from those by reported by Rai et al. (24), who observed complete loss of hemolysis of human erythrocytes upon inactivation or truncation of the carbohydrate-binding domain.

Recently, Levan et al. (25) showed by a combination of biophysical techniques that VCC binds to complex N-glycans with a heptasaccharide GlcNAc2Man2Galβ1–3GalNAc core with nanomolar affinity. The dual preference of VCC for complex N-linked glycans (25) and for O-linked sugars in asialofetuin might be a reflection of the multiple carbohydrate-binding specificities exhibited by β-prism fold lectins (34, 35).

The β-Prism Lectin Domain Makes at Most a Modest Contribution to Membrane Targeting—In agreement with earlier reports (24, 25), the intrinsic tryptophan fluorescence emission and CD spectra of the three VCC variants overlapped (data not shown), suggesting that truncation or mutation of the lectin domain did not affect the solution structure of the hemolysin. The three variants behaved similarly during partitioning in Triton X-114 solution when all of them moved quantitatively to the detergent phase (Fig. 1), indicating that truncation or mutation of the lectin domain did not affect the solution structure of the hemolysin.

Next, we addressed binding of the three hemolysin variants to the rabbit erythrocyte stroma. The situation is more complex than with pure liposomes because the stroma presents a complex architecture of several classes of lipids, proteins, and carbohydrates, allowing multiple modes of interaction to operate simultaneously. Recently, Rai et al. (24) studied interaction of human erythrocytes with VCC and its two variants with no carbohydrate-binding activity at 4 °C. At this temperature, the hydrophobic interactions are not significant, and the only mode of association is carbohydrate-dependent binding of the β-prism lectin domain to stromal glycoconjugates. Here, we used a temperature of 25 °C to ensure conditions comparable to those during pore formation in erythrocytes.
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First, we monitored binding of VCC and its two variants to immobilized rabbit erythrocyte stroma by ELISA (Fig. 3A). As expected, wild-type VCC bound to the stroma. Notably, the VCC variants with a truncated or functionally impaired lectin domain had no apparent affinity for the immobilized stroma. Because VCC variants retained substantial pore-forming activity toward erythrocytes, they must have a mechanism to reach the cell surface. It is more likely that ELISA is not the right technique to assess the contribution of all types of interaction to membrane targeting of VCC.

To include the contribution from hydrophobicity-driven interactions, we had to adopt a strategy that avoids exposure of the stroma-hemolysin complex to detergents. Second, the exposure of a PFT to a protein-lipid bilayer rather than to membrane components would trigger self-assembly of the toxin as a prelude to integration of the toxin complex with the membrane (13). However, it is not clear if membrane vesicles coat on microtiter wells as monolayers or bilayer. Third, the VCC heterotrimer is a strongly hemagglutinating lectin (19) that would cause extensive clumping of either intact erythrocytes or stroma. Collectively, these considerations suggest that neither the conventional ELISA nor the flow cytometry-based assays would be appropriate for quantifying interaction of VCC with the erythrocyte stroma. Here, we allowed VCC and its variants to react with the erythrocyte stroma dispersed in PBS, and the stroma-toxin complex was washed three times with ~100 volumes of PBS at each stage of incubation to minimize nonspecific adsorption of the toxin on the stromal surface.

The bar diagram in Fig. 3B (inset) shows that in comparison with the wild-type toxin, interaction of the two non-carbohydrate-binding variants of VCC with the erythrocyte stroma was not seriously compromised except at a very low toxin/stroma ratio (~1:250). Although various classes of cell surface molecules, along with a heterogeneity in the modes of interactions ranging from carbohydrate-dependent to hydrophobicity-driven, were involved in binding to VCC, an estimate of the values for the association constants by KyPlot yielded values of $3.2 \times 10^7$, $2.6 \times 10^7$, and $1.7 \times 10^7$ M$^{-1}$ for VCC, VCC$^{ΔD617A}$, and VCC$^{50}$, respectively. Although too much could not be read into these data due to the multiplicity of factors involved, values do suggest that the contribution of the β-prism lectin domain to the affinity of VCC for the stroma was significant only at very low toxin concentrations. The role played by carbohydrates as a high affinity receptor in hemolysis by VCC might have some similarity to that of the protein ADAM10 in the hemolysis by Staphylococcus aureus α-toxin (36). Because abrogation of the carbohydrate-dependent interaction affected considerably more the truncated mutant VCC$^{50}$ than the mutant VCC$^{ΔD617A}$ (Fig. 3B), the reduced efficiency of membrane targeting might not be wholly due to absence of carbohydrate-dependent interactions.

Next, we visualized the hemolysin in the stroma-toxin complex by Western blotting (Fig. 3C). Qualitatively, the data are similar to those from Fig. 3B. Interestingly, a significant proportion of stroma-bound VCC$^{50}$ was present as the monomer, in contrast to VCC and VCC$^{ΔD617A}$, which were present almost...
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FIGURE 4. β-Prism lectin domain and oligomerization of VCC in liposome. A, VCC and variants were incubated overnight with PC/CL liposomes at 25 °C. Samples with (+) and without (−) liposome are indicated. The band intensities of the monomer and oligomer were estimated by densitometry to quantify the relative amounts of the two toxin forms. B, a 1:1 molar mixture of VCC and VCC50 was incubated with liposome. The hybrid oligomers were separated by 6.5% SDS-PAGE (shown in the inset). The band intensity of each oligomer was quantified and plotted. The greater efficiency of VCC over VCC50 in forming oligomers in lipid vesicles is reflected in the skewed distribution of intensity in contrast to the symmetrical binomial distribution.

exclusively as the oligomer. It is possible that the reduced efficiency of self-assembly of VCC50 in comparison with wild-type VCC and mutant VCCD617A also contributed to a decrease in efficiency of association with the stroma.

Unexpectedly, asialofetuin and jacalin had little or no impact on binding of VCC to the stroma (data not shown). This was consistent with the observation that preincubation of erythrocytes with the β-prism fold lectin jacalin (37) made the cells more prone to lysis (Fig. 3D). As reported previously (19), when VCC was incubated for 1 h at 25 °C with asialofetuin and examined for hemolytic activity toward rabbit erythrocytes, we observed a sharp fall in the rate of hemolysis (Fig. 3D). The outcome was strikingly different if VCC was added to a red cell suspension containing asialofetuin. There was virtually no inhibition (Fig. 3D), suggesting that asialofetuin is not a competitive inhibitor of interaction of VCC with erythrocyte surface glycoconjugates.

Is It Possible for the Lectin Domain to Play a Carbohydrate-independent Role in Hemolysis?—Next to membrane targeting, the critical event in pore formation is the toxin assembly by circular oligomerization to the β-barrel oligomer (13, 15). Oligomerization of VCC is mediated by the central cytolysin domain that bears sequence and structural homology to the oligomerization and membrane-spanning domain of S. aureus α-toxin (21, 26, 38). To see how the β-prism lectin domain of VCC regulates toxin assembly, we compared self-assembly of VCC, VCCD617A, and VCC50 in PC/CL liposomes (Fig. 4A). The efficiency of oligomerization, expressed as the percent conversion of the monomer to the oligomer, was ~82% for VCC, ~77% for VCCD617A, and a meager ~26% for VCC50. Notably, the efficiency of self-assembly decreased dramatically with loss of the lectin domain but remained virtually unaffected by loss of the carbohydrate-binding function by mutation. The significantly reduced propensity of VCC50 to undergo self-assembly in the lipid bilayer was reflected by the distribution of the relative intensities of the hybrid oligomers, A17, A1B, A1B2, A1B3, A1B4, A1B5, A1B6, A1B7, A1B8, A1B9, A1B10, and B2, (where A and B stand for VCC and VCC50, respectively), formed when an equimolar mixture of VCC and VCC50 was incubated in liposome (Fig. 4B). If the wild-type and truncated hemolysins had equal propensity to oligomerize, the ratio of the intensities of the hybrids would correspond to that of the coefficients in the binomial expansion of \((a + b)^7\). The actual distribution was significantly skewed to the left (Fig. 4B), indicating preferential incorporation of VCC in the hybrid β-barrel assembly. Because liposomes had no carbohydrate, the data indicated that augmentation of toxin assembly by the β-prism lectin domain was independent of its carbohydrate-binding function. This is also supported by the observation that inactivation of the carbohydrate-binding function without disrupting its structure by substitution of Asp617 with Ala had no significant effect on hemolysin assembly (Fig. 4A).

Next, we wondered about the possible mechanism of up-regulation of toxin assembly by the β-prism lectin domain. The crystal structure of the VCC heptamer (26) revealed significant rearrangement of the β-prism domain during toxin assembly, leading to multiple interactions between the cytolysin and lectin domains that are absent in the monomer. A cryo-electron microscopy study (39) of the VCC and VCC50 heptamers indicated that deletion of the β-prism fold caused loss of 7-fold symmetry of the hemolysin assembly. In sum, these data indicated that the presence of the β-prism domain had serious structural consequences for the hemolysin assembly that would be reflected in thermodynamic parameters characterizing the self-assembly of VCC and VCC50.

VCC undergoes self-assembly in water to the β-barrel heptamer, although at a rate much slower than that observed in the lipid bilayer, indicating that lipids are not stringent for oligomerization (20, 33). Interestingly, self-assembly of VCC increased with increasing temperature; by comparison, oligomerization of VCC50 was nearly insensitive to temperature (Fig. 5A). To obtain a rough estimate of the enthalpic and entropic contributions to hemolysin assembly, we incubated VCC in PBS for 16 h at 10, 15, 20, 25, and 30 °C; separated the monomer and oligomer by SDS-PAGE; and quantified the intensity by densitometric scanning (Fig. 5B). The rationale for choosing this temperature range was that there was no gross conformational change in the toxin, as indicated by the near constancy of the ratio \((F_{300}/F_{350})\) of tryptophan fluorescence emission intensities at 320 and 350 nm between 10 and 30 °C (Fig. 5C), so the toxin monomer self-assembled under conditions that roughly simulated pore formation in the target membrane. The protocol was based on the following observations: (a) the monomer-oligomer system reached a steady state within a span of 6 h at 10 °C, after which the relative amounts of the monomer and oligomer did not change with time; (b) the equilibrium was
TABLE 1 Thermodynamic parameters for self-assembly of VCC

| Temperature (K) | $K_a$ ($10^{29} M^{-6}$) | $\Delta G^a$ (kJ M$^{-1}$) | $\Delta H^b$ (kJ M$^{-1}$) | $\Delta S^c$ (kJ M$^{-1}$ K$^{-1}$) |
|----------------|--------------------------|--------------------------|--------------------------|----------------------------------|
| 283            | 1.69                     | -158.24                  | -162.76                  | 173.09                           |
| 288            | 3.45                     | -166.54                  | 115.04                   | 0.96                             |
| 293            | 5.20                     | -173.09                  | 177.26                   |                                  |
| 298            | 23.3                     | -173.09                  | 177.26                   |                                  |
| 303            | 38.8                     | -173.09                  | 177.26                   |                                  |

$^a$ Calculated from $\Delta G^a = -RT \ln K$ (Fig. 5).

$^b$ Calculated from the van't Hoff equation (Fig. 5).

$^c$ Calculated from $\Delta G = \Delta H - T \Delta S$. 

**Figure 5.** Temperature dependence of toxin self-assembly in water. A, VCC and VCC$^{\text{50}}$ were kept in PBS at 25 and 37 °C for 16 h. B, thermodynamics of self-assembly of VCC in water. The VCC monomer (6 μM in PBS) was incubated at 10, 15, 20, 25, and 30 °C for 16 h and subjected to 7.5% SDS-PAGE (inset). The monomer and oligomer concentrations used for calculating the equilibrium constant ($K_a$) for self-association were obtained by densitometric scanning. Table 1 includes unspecified contributions from interaction of VCC with asialofetuin as well as from self-assembly of the toxin, precluding quantitative estimation of thermodynamic parameters for glycoprotein-induced self-assembly of VCC.

Asialofetuin—Next, we studied how a carbohydrate-independent structural role of the β-prism lectin domain during hemolysin assembly was more than compensated by the positive gain in entropy; in other words, structural rearrangement of the carbohydrate-binding domain during toxin oligomerization was a major source of entropy production, which played a significant role in driving self-assembly.

**A Plausible Mechanism for Inhibition of Hemolysis by Asialofetuin**—Next, we studied how a carbohydrate-independent structural role of a β-prism lectin domain in VCC assembly could explain inactivation of the toxin by preincubation with asialofetuin (Fig. 3D). Notably, incubation of the VCC monomer in presence of asialofetuin in PBS induced significant oligomerization (Fig. 6A). The effect increased sharply with an increase in temperature, indicating that the overall process was accompanied by absorption of heat. Because interaction of the VCC β-prism lectin domain is known to be exothermic (24), asialofetuin-induced self-assembly must be strongly endothermic. Therefore, asialofetuin-induced oligomerization was also driven by entropy and opposed by enthalpy. However, the effect included unspecified contributions from interaction of VCC with asialofetuin as well as from self-assembly of the toxin, precluding quantitative estimation of thermodynamic parameters for glycoprotein-induced self-assembly of VCC.

In water and a synthetic lipid bilayer, the β-prism lectin domain promoted toxin assembly by playing a structural role without involving carbohydrate ligands. To see if asialofetuin-induced assembly of VCC has a strict requirement for carbohydrate-dependent interaction with asialofetuin and not merely the presence of the structurally intact but functionally impaired domain, we incubated VCC$^{\text{50}}$ and VCC$^{\text{D617A}}$ with asialofetuin at a 1:2 molar ratio at 30 °C (Fig. 6B) for 6 h. There was no augmentation of assembly for the truncated and mutant toxins in comparison with wild-type VCC, indicating that asialofetuin-induced assembly specifically required carbohydrate-dependent interactions.

Next, we examined the asialofetuin-induced VCC oligomer. It was virtually indistinguishable from the oligomer formed in
water and the lipid bilayer in heptameric stoichiometry, SDS stability, and the characteristic β-barrel morphology in electron micrographs (Fig. 6C). However, the two oligomers differed in one significant aspect that was relevant to the efficiency of the toxin assembly to penetrate the lipid bilayer. The asialofetuin-induced VCC heptamer was soluble in water and tended to move almost exclusively to the aqueous layer in preference to the less polar detergent phase (Fig. 6D). Interestingly, we observed that the asialofetuin-induced VCC oligomer was hemolytically active, with a specific activity of ~6 nM, indicating that it was less potent than the monomer by ~2 orders of magnitude. By comparison, the lipid-induced oligomer resembled a typical integral membrane protein. It tended to precipitate from an aqueous dispersion, required 1% octyl β-glucoside for solubilization, showed almost an exclusive preference for the less polar Triton X-114-enriched phase (Fig. 6D), and was hemolytically inactive, as reported previously (2, 33).

Asialofetuin-induced conversion of VCC to a hemolytically less potent analog of the functionally active VCC heptameric channel formed in a PC/CL bilayer suggests an interesting consequence of the interaction of VCC with cell surface sugars for pore formation. If the resulting oligomer is not competent to insert into the membrane lipid bilayer, it would not contribute to lysis and would correspond to an abortive interaction with the cell surface. Whether cell surface carbohydrates do indeed down-regulate pore formation may actually depend on the relative rates of the two oligomerization events triggered by the two kinds of receptors: carbohydrates (19) and cholesterol (2, 40). In particular, the interaction of VCC with cholesterol-rich lipid microdomains in biological membranes (12) may dominate the pore formation mechanism of VCC and may outweigh a possible inhibitory role exerted by carbohydrates.

Conclusion—The biological role of the lectin domain has been considered, perhaps without exception, in terms of targetting the protein to specific cell surface carbohydrate receptors (41). In this work, we have proposed a novel mechanism for how the lectin-like fold of a β-PFT can regulate its biological function even in absence of a dominant role in membrane targeting. It is likely that the β-prism fold of VCC makes a significant contribution to membrane targeting through carbohydrate-dependent interactions, especially at very low toxin concentrations. However, these interactions are largely swamped by the affinity of the toxin for cholesterol-rich lipid vesicles of synthetic or biological origin (2, 40). The loss of the lectin domain has a more dramatic effect on pore formation than the modest role of carbohydrate-dependent interactions in membrane targeting would suggest. The lectin domain is critical for pore formation because it pushes toxin assembly by contributing to the entropy of oligomerization. It is the lectin domain, rather than the lectin-like function, that is critical for pore formation by VCC.

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