Analysis of Receptor Binding by the Channel-forming Toxin Aerolysin Using Surface Plasmon Resonance*

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Aerolysin is a channel-forming bacterial toxin that binds to glycosylphosphatidylinositol (GPI) anchors on host cell-surface structures. The nature of the receptors and the location of the receptor-binding sites on the toxin molecule were investigated using surface plasmon resonance. Aerolysin bound to the GPI-anchored proteins Thy-1, variant surface glycoprotein, and contactin with similar rate constants and affinities. Enzymatic removal of N-linked sugars from Thy-1 did not affect toxin binding, indicating that these sugars are not involved in the high affinity interaction with aerolysin. Aerolysin is a bilobal protein, and both lobes were shown to be required for optimal binding. The large lobe by itself bound Thy-1 with an affinity that was at least 10-fold weaker than that of the whole toxin, whereas the small lobe bound the GPI-anchored protein at least 1000-fold more weakly than the intact toxin. Mutation analyses provided further evidence that both lobes were involved in GPI anchor binding, with certain single amino acid substitutions in either domain leading to reductions in affinity of as much as 100-fold. A variant with single amino acid substitutions in both lobes of the protein was completely unable to bind the receptor. The membrane protein glycoporphin, which is heavily glycosylated but not GPI-anchored, bound weakly to immobilized proaerolysin, suggesting that interactions with cell-surface carbohydrate structures other than GPI anchors may partially mediate toxin binding to host cells.

Aerolysin is one of the best characterized of the toxins secreted by pathogenic bacteria (1). The protein is released by Aeromonas hydrophila as an inactive 52-kDa precursor called proaerolysin, which is converted to aerolysin by proteolytic removal of a C-terminal peptide (2). Binding of aerolysin to receptors on target cells promotes its oligomerization, and this is followed by membrane insertion and channel formation. Remarkably, all of the proteins that are known to bind aerolysin with high affinity are attached to the cell surface with GPI anchors. These include the erythrocyte aerolysin receptor, brain and T-lymphocyte Thy-1, brain contactin, and VSG of trypanosomes (3–5). We have also shown that cathepsin D, a protein with no affinity for aerolysin, can be converted to an aerolysin-binding protein by addition of a GPI anchor (5), leading to the conclusion that the anchor itself is the major aerolysin binding determinant.

All anchor structures reported to date have a core structure consisting of ethanolamine-HPO₄⁻₆Mana₁–₂Mana₁–₆Mana₁–₄GlcNH₂α₁–₆-myo-inositol-HPO₄ linked to diacylglycerol, alkylacylglycerol, or ceramide. Perhaps not surprisingly, the lipid does not play a role in aerolysin binding; the toxin binds at least as well to GPI-anchored proteins that have been released from cell surfaces by treatment with phosphatidylinositol-specific phospholipase C. The inositol in the core structure can be acylated, and other sugars and/or ethanolamine phosphate can be added to the core mannosyls (7). These additions are not always stoichiometric, adding further complexity to anchor structure (8). Not all GPI-anchored proteins can bind aerolysin, at least in some cases because of species differences in modifications to the core structure. Thus, the Leishmania protease purified with its naturally occurring anchor attached has no affinity for the toxin, whereas the same protein expressed from the cloned gene in a mammalian cell can bind aerolysin.

Proaerolysin and aerolysin both exist as dimers in solution. Based on the crystal structure of the proaerolysin dimer, each monomer is composed of a small globular lobe containing the first 83 residues joined by a short arm of ~10 amino acids to a large elongated lobe containing the remaining residues (9). Electron microscopic analysis of two-dimensional crystalline arrays of the heptameric aerolysin oligomer indicated that part of the large lobe forms an amphipathic β-barrel that becomes the transmembrane channel upon insertion (9, 10), and a number of residues in this lobe that are involved in the oligomerization process have been identified by chemical modification and site-directed mutagenesis (2). The large lobe may also be involved in receptor binding, based on the observation that the α-toxin of Clostridium septicum, which is a homologue of the large lobe of aerolysin, but lacks a region equivalent to the small lobe, can also bind some GPI-anchored proteins (6). However, there is also some evidence that the small lobe is involved in receptor binding. Erythrocytes are far more sensitive to aerolysin than to α-toxin, and fusing the aerolysin small lobe to the clostridial toxin results in a hybrid that is at least as hemolytic as aerolysin (6). The structure of the small lobe also points to a role in carbohydrate binding. We have observed that it is nearly identical to folds in subunits S2 and S3 of pertussis toxin (11), and the common fold, which we have called an aerolysin pertussis toxin (APT) domain, is similar to a fold in C-type lectins and link modules that recognize cell-surface oligosaccharides (11).

Although GPI anchors are clearly the most important determinants of the interaction with aerolysin, there are other prop-
erties of surface proteins that appear to influence toxin binding. For example, we have found that binding to Thy-1 is abolished by protease treatment, indicating that some still uncharacterized feature of the polypeptide chain is required (5). We have also observed that, unlike C. septicum α-toxin, aerolysin binds weakly to glycoporin, a heavily glycosylated membrane protein that is not GPI-anchored (6, 12, 13). Fusing the small lobe of aerolysin to the clostridial protein confers glycoporin-binding ability on the resulting hybrid (6). Taken together, the evidence suggests the possibility that aerolysin may contain two carbohydrate recognition sites: one in the large lobe for GPI anchor binding and a second in the small lobe for binding a second carbohydrate determinant, such as is found in glycoporin (6).

In previous studies, the methods used to estimate aerolysin binding have typically been qualitative. Here we describe the use of surface plasmon resonance to obtain quantitative data on aerolysin binding to GPI-anchored proteins and glycoporphin. The purified large and small lobes of the toxin as well as a panel of proaerolysin variants were used to investigate the role of each lobe in ligand binding.

**EXPERIMENTAL PROCEDURES**

**GPI-anchored Proteins**—Thy-1 was purified from deoxocholate extracts of pig brain using a modification of the procedure of Letarte-Muirhead et al. (14). It migrated as a single band upon SDS-PAGE (see Fig. 4). Contactin was isolated from rat brain according to the following procedure. Fresh brain was homogenized (20%, w/v) in ice-cold 0.16 M NaCl, 50 mM Tris, and 1 mM EDTA, pH 8, containing 1 µg/ml peptatin and 1 mM phenylmethylsulfonyl fluoride. The homogenate (20 ml) was centrifuged at 14,000 × g for 10 min, and the pellet was resuspended in the same buffer and washed by recentrifuging. The washed pellet was resuspended to the original volume, and 4 units of phosphatidylinositol-specific phospholipase C (Sigma) were added. Following incubation at 37 °C for 2 h, the mixture was centrifuged, and the supernatant was applied to a column of immobilized lentil lectin. Fractions containing contactin were eluted from the column with 1 M α-methyl-D-mannoside and applied to a column of DEAE-Sepharose CL-6B (Amersham Pharmacia Biotech) equilibrated with 10 mM Tris, pH 8. Contactin was eluted from the column with a linear NaCl gradient. The pooled fractions gave a single band upon SDS-PAGE and silver staining. In addition, a single N-terminal sequence corresponding to the published sequence of contactin was obtained. Trypanosoma brucei brucei VSG was a generous gift from Dr. Terry Pearson (University of Victoria). More than 95% of the protein migrated as a single band upon SDS-PAGE.

**Deglycosylation of Thy-1**—Deglycosylation was performed using the GlycoShift protein de-N-glycosylation kit from Oxford GlycoSystems (Oxford, United Kingdom). Thy-1 (4 µg) was incubated with 2 units of enzyme in a total volume of 30 µl for 23 h at 37 °C. Deglycosylation was confirmed by SDS-PAGE.

**Proaerolysin Mutants and Truncated Forms of Proaerolysin**—The proaerolysin variants listed in Table I were produced by site-directed mutagenesis as described previously (10). In every case, the desired mutation was confirmed by DNA sequencing. Each proaerolysin variant was purified to homogeneity, and its purity was confirmed by SDS-PAGE. All of the variants described in this study could be correctly processed to aerolysin by trypsin, and once processed, they were all capable of forming heptameric oligomers in the same way as native aerolysin. Many of these changes were found to have no effect on the activity of the protein, whereas others reduced activity by affecting the ability of the toxin to oligomerize or insert (2). Several variants, each with a change at the top of the large lobe, had lower activity than the wild type, and the decrease could not be attributed to changes in oligomerization or insertion. These variants are included in Table I. One of them, C159S, has a free cysteine at 164 and has lost the 159–164 disulfide bridge of the native toxin. Also included in Table I is the variant Y61A/W324A. This variant had no detectable hemolytic activity (Table I), nor was it able to kill T-lymphoma cells, even at concentrations 105-fold higher than the wild type (data not shown).

**Affinity of Native Aerolysin and Proaerolysin for Different GPI-anchored Proteins**—Similar toxin binding profiles were observed for the three GPI-anchored proteins (Thy-1, VSG, and contactin) that were tested as ligands (Fig. 2 and Table II). In addition, there were no significant differences in the kinetics of proaerolysin and aerolysin binding to Thy-1 and contactin. With Thy-1 surfaces, the amount of toxin binding increased after the initial acid regeneration step (Fig. 2), but remained stable throughout subsequent binding and regeneration cycles. A slower dissociation rate constant was responsible for the improved binding on acid-regenerated Thy-1 surfaces (Table II). The rate constants given in Table II were derived by fitting the data to a 1:1 interaction model. In all instances, the fitting was not ideal, but was acceptable if only the early dissociation data were used. The Thy-1 data gave better fitting to this model than the VSG and contactin data and even met the rigorous demands of global fitting (Fig. 3 and Table I). The analyses obtained for the proaerolysin/Thy-1 interaction by averaging the rate constants derived by local fitting of individual sensor-
gram data and by global fitting were similar (Table II).

**Binding of Proaerolysin Variants and the Small and Large Lobes**—Since the data for toxin binding to Thy-1 fit better to a 1:1 interaction model than those collected on VSG and contactin surfaces, Thy-1 surfaces were used for comparison of the ligand binding affinities of wild-type, truncated, and mutant forms of proaerolysin. High density surfaces were required for these comparisons in order to obtain acceptable signals with the lower affinity binders. Local fitting was used in data analysis since the use of high density surfaces gave data that did not always meet the demands of global fitting. Both lobes of proaerolysin were required for full binding activity on Thy-1 surfaces, and binding to Thy-1 was observed with isolated small and large lobes (Table I). The small lobe (domain 1) bound weakly to Thy-1. The data were amenable only to Scatchard analysis because of the rapid kinetics (Fig. 4). Relative to the whole toxin, the $K_d$ for binding of the large lobe (domains 2–4) to Thy-1 ($8 \times 10^{-7}$M) was $>50$-fold weaker, and that of the small lobe ($5.5 \times 10^{-5}$M) was $5000$-fold weaker (Table II).

Analysis of mutant binding to immobilized Thy-1 identified several residues in domain 1 and at least one residue in domain 2 that appear most important in ligand binding. The affinities

**TABLE I**

| Proaerolysin form | Native Thy-1 | Deglycosylated Thy-1 |
|-------------------|--------------|----------------------|
| $k_a$  $M^{-1}s^{-1}$ | $k_d$  $s^{-1}$ | $K_a$  $\mu$ | Relative binding | $k_a$  $M^{-1}s^{-1}$ | $k_d$  $s^{-1}$ | $K_a$  $\mu$ | Relative binding | Hemolytic activity |
| Wild type | $6.9 \times 10^4$ (12%) | $7.5 \times 10^{-4}$ (17%) | $1.1 \times 10^{-4}$ (12%) | 100 | $3.1 \times 10^4$ (14%) | $7.8 \times 10^{-4}$ (1%) | $2.6 \times 10^{-4}$ (16%) | 100 | 100|
| Small lobe | 5.0 | $10^{-5}$ | 0.02 | 3.7 | $10^{-5}$ | 0.07 | 0 | 3.7 | 0.07 |
| Domains | $3.3 \times 10^3$ (5%) | $2.6 \times 10^{-3}$ (2%) | $8.0 \times 10^{-7}$ (5%) | 1.4 | 6.3 | $10^3$ (16%) | $2.2 \times 10^{-3}$ (1%) | $3.5 \times 10^{-3}$ (15%) | 7.4 | 0.5 |
| W45A | $3.9 \times 10^3$ (29%) | $1.7 \times 10^{-3}$ (5%) | $4.5 \times 10^{-3}$ (23%) | 2.4 | 3.9 | $10^3$ (30%) | $1.2 \times 10^{-3}$ (21%) | $3.2 \times 10^{-3}$ (21%) | 8.1 | 2 |
| I47E | 1.1 | $10^3$ (34%) | $2.6 \times 10^{-3}$ (24%) | $2.9 \times 10^{-6}$ (57%) | 0.4 | 2.0 | $10^3$ (16%) | $8.1 \times 10^{-4}$ (10%) | $4.1 \times 10^{-4}$ (25%) | 63 | 1 |
| M57A | 1.4 | $10^4$ (23%) | $1.7 \times 10^{-3}$ (13%) | $1.3 \times 10^{-7}$ (13%) | 8.4 | 5.9 | $10^3$ (36%) | $1.3 \times 10^{-3}$ (8%) | $2.3 \times 10^{-3}$ (28%) | 11 | 3 |
| Y61A | 5.9 | $10^4$ (20%) | $1.5 \times 10^{-3}$ (6%) | $2.6 \times 10^{-7}$ (14%) | 4.2 | 5.2 | $10^4$ (48%) | $8.5 \times 10^{-4}$ (11%) | $1.9 \times 10^{-4}$ (77%) | 13 | 1 |
| K88Q | 5.4 | $10^3$ (19%) | $2.0 \times 10^{-3}$ (12%) | $3.7 \times 10^{-7}$ (13%) | 3.0 | 4.3 | $10^3$ (32%) | $1.4 \times 10^{-3}$ (13%) | $3.4 \times 10^{-3}$ (32%) | 7.6 | 1 |
| N154D | $6.4 \times 10^4$ (18%) | $1.1 \times 10^{-3}$ (2%) | $1.7 \times 10^{-8}$ (18%) | 65 | 5.7 | $10^4$ (39%) | $1.2 \times 10^{-3}$ (11%) | $2.3 \times 10^{-3}$ (27%) | 117 | 25 |
| C150S | $8.6 \times 10^4$ (39%) | $8.3 \times 10^{-3}$ (3%) | $1.1 \times 10^{-7}$ (35%) | 10 | 3.9 | $10^4$ (14%) | $5.7 \times 10^{-3}$ (5%) | $1.5 \times 10^{-3}$ (16%) | 17 | 6 |
| Y162A | $6.0 \times 10^4$ (18%) | $1.6 \times 10^{-3}$ (13%) | $2.8 \times 10^{-8}$ (32%) | 39 | 5.0 | $10^4$ (25%) | $1.7 \times 10^{-3}$ (10%) | $3.6 \times 10^{-3}$ (15%) | 72 | 6 |
| K167M | $3.9 \times 10^4$ (10%) | $6.5 \times 10^{-4}$ (11%) | $1.7 \times 10^{-8}$ (8%) | 65 | 2.6 | $10^4$ (28%) | $6.6 \times 10^{-3}$ (21%) | $2.6 \times 10^{-3}$ (7%) | 100 | 25 |
| K171C | $5.6 \times 10^4$ (13%) | $1.0 \times 10^{-3}$ (22%) | $1.7 \times 10^{-8}$ (12%) | 65 | 4.2 | $10^4$ (24%) | $1.0 \times 10^{-3}$ (7%) | $2.4 \times 10^{-3}$ (24%) | 108 | 100 |
| W324A | $5.8 \times 10^4$ (23%) | $2.0 \times 10^{-3}$ (4%) | $3.6 \times 10^{-7}$ (24%) | 3.1 | 3.3 | $10^4$ (44%) | $1.5 \times 10^{-3}$ (8%) | $5.8 \times 10^{-3}$ (47%) | 4.6 | 12.5 |
| H332N | $4.0 \times 10^4$ (14%) | $1.6 \times 10^{-3}$ (2%) | $4.1 \times 10^{-8}$ (15%) | 27 | 3.4 | $10^4$ (18%) | $1.7 \times 10^{-3}$ (6%) | $5.1 \times 10^{-3}$ (15%) | 51 | 25 |
| E407C | $6.1 \times 10^4$ (18%) | $8.5 \times 10^{-4}$ (14%) | $1.3 \times 10^{-8}$ (8%) | 85 | 4.2 | $10^4$ (34%) | $1.1 \times 10^{-3}$ (7%) | $2.8 \times 10^{-3}$ (30%) | 93 | 100 |
| Y61A/W324A | NA | NA | NA | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

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**a** Values in parentheses are S.D. values based on analyses at five or more concentrations.

**b** As percent of wild-type activity.

**c** Determined by Scatchard analysis.

**d** Not active.

Fig. 1. Stereo view of the proaerolysin dimer showing key residues involved in GPI anchor binding. The disulﬁde bridge between residues 159 and 164 is also shown, as is Lys-171, which may be involved in glycophorin binding (see “Results”). The diagram was prepared using SETOR (20).
of the five domain 1 variants (W45A, I47E, M57A, Y61A, and K66Q) were each <5% of the wild-type activity (Table I). In all instances, slower association and faster dissociation rates contributed to the lower affinities. Of the domain 2 variants, only W324A reduced ligand binding affinity to a level that was <5% of the wild-type activity. Of the lower affinity mutants, W324A was unique in that weaker binding was attributable entirely to a faster dissociation rate (Table I). A double domain 1/domain 2 mutant (Y61A/W324A) was observed to be completely inactive.

There was generally excellent agreement between the affinities determined by surface plasmon resonance and the hemolytic activities of the various forms of proaerolysin (Table I). For example, relative to the wild-type protoxin, the affinity and lytic activity of W45A were reduced ~50- and 75-fold, respectively (Table I). The small and large lobes were both inactive in the titer assay at the concentrations we used. This is consistent with the fact that both lobes are presumably required not only for binding, but for the optimal formation of the insertion-competent oligomer.

Proaerolysin Binding to Deglycosylated Thy-1—Thy-1 was efficiently de-N-glycosylated by enzymatic treatment. SDS-PAGE showed a somewhat diffuse band at ~29 kDa for native Thy-1 and a sharp band at ~15 kDa for enzyme-treated Thy-1 (Fig. 5). The profiles for the binding of wild-type whole toxin as well as the truncated and mutant forms to deglycosylated Thy-1 were similar to those observed for native Thy-1 binding (Table I). With the exception of mutant I47E, which bound 10-fold more strongly to the deglycosylated form than to the native form, all truncated and mutant forms of proaerolysin bound to deglycosylated Thy-1 with kinetics and affinities that were very similar to those observed for binding to native Thy-1.

Binding Stoichiometries—Analyte concentrations that were sufficiently high (at least $10 \times K_D$) to result in near saturation of various surfaces with analyte were used in order to derive information relating to the stoichiometries of toxin/ligand interaction under the experimental conditions employed here. Aerolysin was used at a lower concentration than proaerolysin because it begins to oligomerize at concentrations higher than 200 nM. With Thy-1 surfaces, relatively little of the Thy-1 ligand was available for toxin binding. Toxin/Thy-1 ratios were much lower than 1, based on monovalent binding for both monomeric large lobe and dimeric proaerolysin and aerolysin. In addition, the toxin/Thy-1 ratios were lower for the large lobe relative to proaerolysin and aerolysin and also for deglycosylated Thy-1 relative to native Thy-1 surfaces (Table III).

The binding stoichiometries were also determined using thiol-immobilized K171C, a proaerolysin mutant with wild-

TABLE II

| Immobilized ligand       | Curve fitting | Proaerolysin | Aerolysin |
|--------------------------|---------------|--------------|-----------|
|                          | $k_a$          | $k_d$        | $K_D$     | $k_a$      | $k_d$        | $K_D$     |
| Thy-1                    | Local         | $3.3 \times 10^4$ | $2.1 \times 10^{-3}$ | $6.6 \times 10^{-8}$ | ND$^a$ | ND | ND |
| Acid-treated Thy-1       | Global        | $3.9 \times 10^4$ | $7.7 \times 10^{-4}$ | $2.0 \times 10^{-8}$ | $2.4 \times 10^4$ | $4.5 \times 10^{-4}$ | $1.9 \times 10^{-8}$ |
| Acid-treated Thy-1       | Local         | $5.0 \times 10^4$ | $8.3 \times 10^{-4}$ | $1.8 \times 10^{-8}$ | ND | ND | ND |
| Acid-treated contactin   | Local         | $2.0 \times 10^4$ | $6.0 \times 10^{-4}$ | $3.8 \times 10^{-8}$ | $2.8 \times 10^4$ | $3.6 \times 10^{-4}$ | $1.3 \times 10^{-8}$ |
| Acid-treated VSG         | Local         | $1.0 \times 10^4$ | $2.3 \times 10^{-3}$ | $2.8 \times 10^{-8}$ | ND | ND | ND |

$^a$ Not determined.
Specificity and Kinetics of Aerolysin Binding


type activity (Table III). In this orientation, both chains of proaerolysin bound Thy-1. The ratio of bound Thy-1 to immobilized dimer was 1.8 at an analyte concentration of 2 μM. The kinetics of Thy-1 binding to proaerolysin surfaces were similar to those observed in the other orientation (data not shown).

**Glycophorin Binding to Proaerolysin**—Attempts at immobilizing glycophorin on CM5 sensor chips were unsuccessful, presumably because of the low pI of the protein. To use alternative methods of monitoring the interaction of proaerolysin and glycophorin, two proaerolysin variants with introduced cysteines (E407C and K171C) were used. Both of these variants had similar activities and Thy-1 affinities compared with the wild type (Table I). The K171C variant was readily immobilized on sensor chips by thiol coupling, but attempts at immobilizing E407C in this way were not successful. However, both variants could be captured on streptavidin surfaces following biotinylat-

![Fig. 4. Scatchard plots for the determination of proaerolysin affinity for native (open squares) and deglycosylated (closed squares) Thy-1.](image)

![Fig. 5. Deglycosylation of Thy-1. Shown are the results from SDS-PAGE of molecular weight markers (lane A), deglycosylated Thy-1 (lane B), and native Thy-1 (lane C).](image)

**Table III**

Interaction stoichiometries of proaerolysin, aerolysin, and domains 2–4 with Thy-1

| Ligand on chip | Analyte | Analyte/ligand |
|----------------|---------|---------------|
| 1000 RU native Thy-1 | 4 μM proaerolysin | 0.22 |
| 1000 RU native Thy-1 | 200 nM aerolysin | 0.26 |
| 1000 RU native Thy-1 | 8 μM domains 2–4 | 0.15 |
| 1000 RU deglycosylated Thy-1 | 2 μM proaerolysin | 0.06 |
| 1750 RU proaerolysin K171C | 2 μM native Thy-1 | 1.8 |

![Fig. 6. Binding of glycophorin to proaerolysin. Glycophorin (20 μM) was injected over 3000 RU of biotinylated E407C captured on a streptavidin surface.](image)

**DISCUSSION**

The results of the present quantitative analysis of aerolysin binding using surface plasmon resonance allow us to draw several important conclusions about the nature of the interaction between the toxin and its receptors. Perhaps most important, the results show that both the small lobe of the toxin and a region at the top of the large lobe are involved in binding to GPI anchors. This conclusion is consistent with the circumstantial evidence we had before we began this study. Thus, we had observed that the small lobe of the toxin has a fold that we have called the APT domain (Fig. 1), which is similar to the carbohydrate recognition domain of the C-type lectins and other proteins. Changing any of the five conserved surface-exposed residues in the aerolysin APT domain had a profound effect on binding to Thy-1 (Table I). This was due to a decrease in the interaction of the small lobe with the glycosyl portion of the anchor, rather than with the N-linked sugars of Thy-1, since proaerolysin and aerolysin bound at least as well to de-N-glycosylated Thy-1 as to the native glycoprotein. Changing residues in the small lobe that are not conserved in the APT fold had little or no effect on binding (data not shown).

The observation that *C. septicum* α-toxin also binds GPI-anchored proteins, despite the fact that it lacks a region homologous to the small lobe of aerolysin, suggested that the large lobe is also capable of binding the anchor, and this, too, was confirmed in this study. In fact, the results in Table I show that the large lobe by itself bound better to Thy-1 than the purified small lobe. This interaction appears to involve the top of the large lobe, in the region we have called domain 2. Removing the disulfide bridge by replacing Cys-159 with serine or changing a number of nearby residues, most notably Tyr-162 and Trp-324 (Fig. 1), all reduced aerolysin binding to Thy-1. It
should be noted that we have changed >40 other residues throughout the large lobe with no apparent effects on receptor binding, more evidence that it is the top of the large lobe that contributes to binding.

In common with other carbohydrate-binding proteins, the GPI-binding site of aerolysin contains numerous aromatic residues. In carbohydrate-binding proteins, the side chains of aromatic amino acids frequently stack against the hydrophobic faces of sugar rings (18). Clearly, two proaerolysin residues (Tyr-62 in the small lobe and Trp-324 in the large lobe) are crucial for binding since the double mutant Y61A/W324A was inactive. These residues are close to each other in the aerolysin dimer, raising the possibility that initial toxin binding to individual GPI anchors may involve both monomers. The mutation studies indicated that a third aromatic residue, Trp-34, was also a component of the ligand-binding site. The significant clustering of aromatic residues on the surface of domain 2 (9) suggests a probable role for additional domain 2 residues in binding. The origin of the dramatic effect of the I47E mutation on binding (Table I) is not clear, but may relate more to the introduction of a negative charge than to alteration of the ligand-binding site. Significantly, the effect was much more pronounced with native Thy-1 than with deglycosylated Thy-1. With native Thy-1, this mutation reduced the affinity less than that of the large lobe. Also, the effect of the C159S mutation (Table I) may not reflect direct alteration of a ligand contact residue, but may be due to the loss of the 159–164 disulfide bridge.

With a $K_D$ in the vicinity of 20 nM, the interaction of aerolysin with the Thy-1 anchor is very high affinity for protein-carbohydrate bind

The core GPI structure has two phosphate groups (7) that could interact ionically with the toxin and contribute to the unusually high affinity. The observation that the K66Q mutation reduced proaerolysin affinity for Thy-1 (Table I) supports this possibility. It has been observed that antibodies specific for oligosaccharides containing 3-deoxy-d-manno-2-octulosonic acid also have affinities in the nanomolar range, and this has been attributed to the presence of charged groups on the antigens.4

The dissociation kinetics indicated monovalent binding of proaerolysin and aerolysin dimers to immobilized Thy-1. The avidity effect associated with bivalent binding would result in dramatically slower dissociation rate constants, and the $k_d$ for the interaction of monomeric large lobe with native Thy-1 was only 3-fold faster than that of the dimeric protein (Table I). Also, the binding stoichiometries were consistent with monovalent binding of the bivalent toxin molecules. The reasons for the low toxin/Thy-1 ratios on Thy-1 surfaces under near saturation conditions are not clear, but are presumably related to poor accessibility of the GPI anchor to the toxin. It is possible that Thy-1 molecules are immobilized in an orientation that obstructs binding. Alternatively, accessibility may be affected by Thy-1 aggregation, through the lipid tails, on the sensor chip surfaces. Interestingly, Thy-1 accessibility was less restricted with native Thy-1 relative to deglycosylated Thy-1 and for intact forms of the toxin relative to the large lobe.

Our results also confirm that aerolysin can bind to erythrocyte glycoporin, although the interaction is considerably weaker than the interaction with any of the GPI-anchored proteins. The fact that the K171C variant was unable to bind glycoporphin, although it could bind Thy-1, indicates that the binding site for sugars of the erythrocyte protein is at least partly different from the binding site for the GPI anchor. It is possible that there is an ionic interaction between the lysine side chain at position 171 of the toxin and the acidic sugars displayed on glycoporphin. Residue 171 is located in domain 2, with its side chain pointing away from the surface of proaerolysin opposite the interface between the two chains (Fig. 1). It is in relatively close proximity to domain 1 of the other chain of the dimer. Domain 1 has been previously implicated in glycoporphin binding (10). Interestingly, the region of domain 1 of the adjoining chain that is closest to Lys-171 has two lysines at positions 66 and 67 (Fig. 1). In the assays performed here, any interaction between proaerolysin and the N- and O-linked sugars on Thy-1 would be completely masked by the strong binding to the GPI anchor. However, it is possible that the initial interaction of the toxin with host cells involves carbohydrate structures more distant from the membrane surface than GPI anchors, such as the N-linked sugars of Thy-1 and the sugars of glycoporphin. Such a mechanism for binding might explain how aerolysin penetrates the cell’s glyocalyx, and it would be analogous to that observed in the inflammatory response wherein the low affinity selectin/oligosaccharide interaction is followed by a stronger interaction between cell adhesion molecules and integrins (19). In addition, it would explain the dramatic increase in hemolytic activity observed when the small lobe of aerolysin, itself inactive, is fused to the weakly hemolytic C. septicum α-toxin.

Acknowledgments—We thank Rebecca To and Dr. S. V. Evans for generating the stereo diagram of proaerolysin. The technical assistance of Tracy Lawrence, Kim Nelson, and Miranda Tradewell is also gratefully acknowledged.

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